Impaired Organ Perfusion

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Impaired Organ Perfusion: Assessment of Early Diagnosis and Interventional Strategies

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"Impaired Organ Perfusion: Assessment of Early Diagnosis and Interventional Strategies"

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Cover: Bright field microscopy of University of Wisconsin–induced branched red blood cell rouleaux networks; magnification 500×.

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"Declare the past, diagnose the present, foretell the future; practice these acts. As to diseases, make a habit of two things: to help, or at least to do no harm."

Hippocrates, *Epidemics*.

Celor dragi

Contents

Chapter 1

Introduction

1.1 General considerations

Understanding Quality of Life is nowadays of critical importance when providing health care. Current medical practices and the expanding medical technology lay their fundamental principles on prolonging Life at all costs. Decisions on what research or treatments should invest in are closely related to their effect on patient's Quality of Life. Conventional medical indicators of Life Quality are the rate of cure, disease–free survival, side effects, and costs. In order to build a correct diagnosis and treatment decision, the mentioned factors are taken into consideration together with indicators of the impact of patient's personality on the disease, the level of satisfaction, and the general health condition. The work described in this thesis aims to mediate some of the today's clinical controversies, to challenge some of the standard decisions in the current clinical practice by pointing out both weak and strong points in the algorithm of diagnosis and treatment of organ injury and dysfunction during disease.
Organ perfusion is a generic physiological term that refers to the process of oxygen and nutritive delivery of arterial blood to the capillary bed in the biological tissue of specific organs. A compromised organ perfusion might have a multifactorial etiology: low cardiac output caused by cardiogenic or hypovolemic shock[1], acute respiratory

failure[2], low blood oxygen–carrying capacity[3], macro/micro–vascular collapse[4,5], sepsis with systemic inflammatory response[6,7], hypotension and hypoxia as secondary insults to brain injury[8,9]. In our approach, we investigated the organ perfusion and subsequent organ viability during acute organ support, as performed during open heart surgery with cardiac arrest and cardiopulmonary bypass, and during donor management and organ procurement prior to transplantation.

1.2 Theoretical background

Acute organ support by means of cardiopulmonary bypass

The father of surgical cardiopulmonary bypass for humans was John H. Gibbon, Jr, MD.[10], whose interest in and determination to develop cardiopulmonary bypass arose in 1931 when he sat at the bedside of a young woman who was dying of a pulmonary embolus.

> "It is only necessary to ... withdraw blood from a vein, introduce oxygen and allow carbon dioxide to escape, and then inject the blood into a peripheral artery. It would permit, of course, operations within the heart under direct vision."
>
> *John Gibbon Jr., 1949.*

He and his wife, Mary Gibbon, devoted the better part of their lives to the laboratory development of a usable pump and oxygenating system. On May 6, 1953, John Gibbon made surgical history by using his apparatus for the successful repair of an atrial septal defect in an 18 years old woman[11]. Although he subsequently operated on 2 more patients, both died; he was so disappointed that he never again performed an open heart operation. However, by the mid–1950s, the first successful clinical procedures had been reported[12]. Although it was obvious that the equipment was primitive and risks were high, the practicality of developing effective treatments for congenital and valvular heart disease had been demonstrated.

The cardiopulmonary bypass (CPB), or heart–lung machine, is an apparatus through which blood is temporarily diverted, especially during open heart surgery, to be oxygenated and pumped through the body, maintaining circulation until the heart and lungs are able to return to normal functioning[13]. The heart–lung machine allows open–heart surgery to be performed on an arrested heart without the patient suffering from hypoxia. Additional to open heart surgery, extracorporeal circulation is a promising adjunct to surgical techniques in neurosurgery, thoracic aortic surgery, complex lung resections, and tumor surgery[14]. Last but not least, mechanical systemic circulatory support is used successfully during bridging to transplantation, to

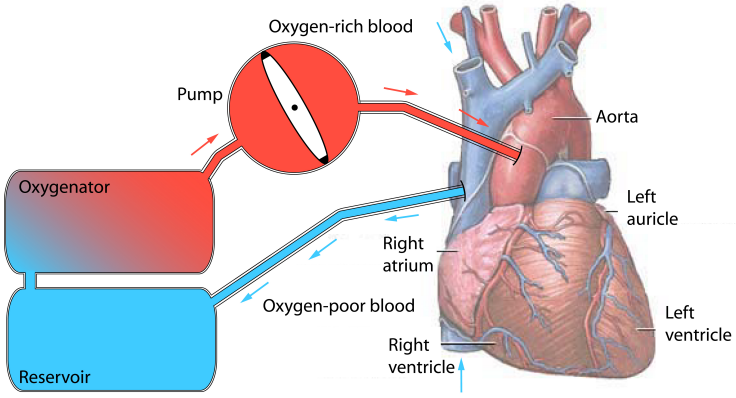

Figure 1.1: The principle of the heart–lung machine.

support life in recipient patients waiting for transplantation or to maintain organ function until organ procurement in transplant donors[15].
Cardiopulmonary bypass is accomplished by the use of an extracorporeal circuit and a pump. The standard CPB circuit (Fig. 1.1) consists of connective tubing, a blood reservoir, oxygenator, heat exchanger, and filter. A venous cannula is placed in the vena cava or right atrium to drain unoxygenated blood by gravity through connective tubing into a blood reservoir. The blood is then pumped to an oxygenator where oxygenation and carbon dioxide removal takes place. Blood temperature may be adjusted by the use of a heat exchanger. Generally, whole body hypothermia is induced while the patient is on CPB. Hypothermia decreases the body oxygen consumption and allows lower blood delivery rates. After the surgical correction is performed, the blood temperature is rewarmed. The blood is filtered to reduce the potential for an embolism and is pumped back to the body through an arterial cannulation. Prior to initiation of CPB, the extracorporeal pump circuit is primed with a balanced crystalloid/colloid solution (e.g. hydroxyethyl starch HES solutions). Once CPB has been initiated, the heart is allowed to beat or it may be temporarily arrested by the administration of cardioplegia solution. Cardioplegia is a cold crystalloid or blood solution that contains a high concentration of potassium. The potassium is used to induce cardiac arrest. The cold temperature of the cardioplegia solution (4° C) along with other drugs in the solution reduces the oxygen requirements of the myocardium and helps to preserve the heart during the ischemic arrest period.
Contact of blood components with the artificial surface of the bypass circuit, aortic cross–clamping, cardioplegic techniques, low perfusion pressures with a non–physiologic profile (continuous), hemodilution, allogenic blood transfusion, and operative trauma result in a systemic inflammatory response syndrome (SIRS) and ischemia/reperfusion

injuries, generally acknowledged as a "post CPB syndrome". The systemic inflammatory response in combination with the ischemia/reperfusion injuries and the multiple gaseous, lipoprotein and particulate emboli generated during CPB result in organ dysfunction affecting the heart, brain, lungs, kidneys and intestine.
Special clinical attention is addressed to the pathophysiology of the systemic inflammatory response syndrome during CPB. The complex inflammatory reaction set in motion during exposure of blood to large areas of synthetic materials involve activation of complement, platelets, neutrophils, monocytes and macrophages triggering the coagulation, fibrinolytic and kallikrein cascades and increasing blood concentrations of interleukins, tumor necrosis factor, leukotriens. A subsequent increase in endothelial cell permeability allows transvascular migration of activated leukocytes into the tissues with additional vascular and parenchimal damage[16–18].
Preoperative administration of corticosteroids, with methylprednisolon and dexamethasone being the two most utilized agents, has been demonstrated to inhibit the activation of the plasmatic and cellular inflammatory response[16], to decrease the pro– to anti–inflammatory interleukins ratio[19], and to minimize tissue edema[20].
Over the last 50 years increased understanding of the physiology and pathophysiology of the response to CPB has led to steady improvements in circuit design and a reduction in postoperative morbidity. The development of more biocompatible polymeric materials[21], arterial in–line filters[22], membrane oxygenators[23], and centrifugal blood pumps[24] have resulted in less intraoperative hemolysis, less blood activation, and decreased circulation of emboli[25]. Surface modification of circuits with heparin[26,27] to mimic the native circulatory system and the use of pharmacological agents such as aprotinin[28] have been shown to attenuate blood activation, systemic inflammation, and organ dysfunction[29,30].
Even with these developments, post–CPB inflammation, ischemia/reperfusion injury, organ injury and organ dysfunction are attenuated but not completely inhibited[31–33].
The use of mechanical assistance as a bridge to transplantation or a bridge to recovery are presently the main indications for mechanical circulatory support in transplant recipients, allowing a prolonged survival with a reasonable Quality of Life. Special attention is directed to transplantation in infants and children, where the problem of organ donor shortage is even worse when compared to the situation in the field of adult heart transplantations. Newly developed pulsatile, paracorporeal ventricular assist devices designed for long–term assist in children have demonstrated their ability to provide excellent results beyond the abilities of extracorporeal membrane oxygenation and centrifugal pumps, which are still the mainstay of mechanical support in children worldwide[34].

Pathophysiology of impaired tissue perfusion and donor management in organ transplantation

Transplantation has been incorporated into the treatment of patients with end–stage diseases of most major organ systems in recent years. However, organ supply is the greatest limitation to organ transplantation, and thus good management of available donors is a high priority. The majority of donors are heart beating, brain dead cadaveric donors from whom multiple organs are procured for transplantation. There are several steps on the path from potential donor to actual donor. These steps have been defined as: identification and detection of all potential donors; brain death determination; approaching potential donor families for consent; and donor medical management[35]. Due to brain death and loss of integrated neurological function, the potential organ donor manifests major physiologic derangements that require aggressive, labor–intensive management throughout the hospitalization until organ procurement or cessation of life support in order to maintain organ function. Experimental and clinical brain death studies define as major pathophysiologic mechanisms the vascular regulation injury and metabolic cellular injury[36]. Myocardial dysfunction and impaired systemic vascular tone trigger hemodynamic instabilities leading to impaired inotropy and chronotropy, dysrhythmias, decreased cardiac output and hypotension[37–39]. Arginin–vasopressin production in the pituitary tract generally ceases after brain death, which commonly leads to diabetes insipidus[40]. The loss of free water caused by diabetes insipidus may lead to dehydration, hypovolaemia, and blood electrolyte abnormalities. Hypokalaemia, hypocalcaemia, hypophosphataemia, and hypomagnesaemia may contribute furthermore to decrease in cardiac function and organ blood flow[41].

Endothelial activation in brain dead donors has gained lately considerable attention in the scientific discussion around the pathology of brain death prior to organ retrieval. The endothelium is considered to be a dynamic interface between the vascular compartment and the extravascular space regulating protein flux, local blood flow, coagulation cascade and the trafficking of the inflammatory cells from the blood into tissue. The vascular endothelial phenotype changes dramatically under pathophysiologic conditions with regards to expression of cell adhesion molecules, cytokines, and substrates that promote thrombosis and inflammation. In this respect, one of the earliest events that was demonstrated to develop after brain injury is the expression of a series of adhesion molecules in sequence by activated vascular endothelium[42,43]. Additionally, an immune activation with increased endothelial cell activation and immediate early gene expression is shown to occur after brain death induction[44]. The expression of endothelial adhesion molecules (intercellular adhesion molecule–1 and vascular cell adhesion molecule–1) and the influx of leukocytes in the kidney occurs faster and is more profound when hemodynamic instability in the brain dead donor is not corrected[45]. In this way, the involvement of the activated vascular endothelium was linked incontestably to the progression of inflammation after brain death.

Donor management has been considered the most neglected area of transplant medicine. In support for this affirmation stand statistical data showing that failure to provide adequate physiological support to potential donors accounts for at least 25% of lost donor organs, and that adoption of an aggressive protocol for donor management, including both intensive monitoring and therapy, has allowed the donor retrieval rate to increase by approximately 30%[46–48].

All organs may benefit from aggressive management. Current evidences regarding the evaluation and management of potential donors led to the recommendation that organ procurement organizations should use a standard protocol for donor management which should include application of pulmonary arterial catheterization[49,50].

A Critical Pathway for the Organ Donor was described, including five distinct, but often overlapping phases: Phase I, Donor Referral; Phase II, Declaration of Brain Death and Consent; Phase III, Donor Evaluation; Phase IV, Donor Management; and Phase V, Organ Recovery[51]. Each phase has five subsections that the ICU staff and/or the organ procurement coordinator can use as a guide for thoroughness of evaluation and management. These five subsections are: General Management; Laboratory and diagnostic Tests; Respiratory Therapy; Treatments; Intravenous Fluids and Medications. The Critical Pathway is designed to provide the information necessary to evaluate the functional status of the kidneys, liver, pancreas, heart and lungs and to determine the management steps which need to be taken to improve and optimize the performance of each organ. In addition to an invasive monitoring, these patients need a meticulous attention to their hemodynamic variables.

The early administration of desmopressin to treat diabetes insipidus, differentiated use of fluid resuscitation and distinct catecholamine support are special features of an appropriate basic treatment. Hormonal resuscitation (thyroxin, vasopressin, insulin) has been reported to stabilize and improve cardiac function in brain dead donors. The administration of corticoids has to be considered if a sufficient circulation can not be regained[52].

As the management of organ donor patients becomes more complex, recovery coordinators often have to change their thinking and resort to extra, nonconventional means of diagnosis and management. The standard laboratory diagnostic tests are not always early, specific and sensitive enough, so addition of new biomarkers to diagnose organ injury is needed. Furthermore, instead of aggressive pharmacologic interventions often implemented in an attempt to stabilize donor hemodynamics, the addition of an extracorporeal circulatory assist device might prove beneficial in optimizing organ perfusion. There are reports in literature showing that cardiopulmonary bypass and profound hypothermic circulatory arrest may be easily combined with traditional procurement flushing techniques, providing excellent organ preservation for subsequent transplantation. This approach could optimize organ recovery from hemodynamically unstable donors, increasing the number of organs available for transplantation[53].

Organ procurement: wash–out and preservation procedures prior to transplantation

In the 1930s, the classical experiments of Carrel and Lindbergh included perfusion techniques to preserve organs for transplantation. They established ground rules for organ preservation by continuous ex–vivo perfusion; expert technology, perfect asepsis, and controlled biological conditions. The Spanish Civil War marked the advent of blood banks and clinical application of tissue storage techniques. Cold storage was used to diminish metabolic demand. Fortunately blood, the first widely preserved and transplanted biological substance, lent itself well to extended storage. Refrigerated storage was possible for 3 weeks.
The discovery of cryoprotectants by Polge and coworkers in 1949 ushered in a major extension of preservation times for a variety of simple cells and tissues. Blood cells and gametes (even embryos) could be stored after freezing. Weeks or months later, they could be thawed and successfully reimplanted as autografts or allografts. Such freezing was only successful for isolated cells or undifferentiated multicellular embryos: complex and heterogeneous organs were highly sensitive to freezing damage. The biophysical problems of freezing large organs were subsequently defined by Pegg and other workers. To date no consistent success has been obtained with the use of freezing to preserve organs such as kidneys, liver, or hearts. Concepts of frozen humans waiting revival at an appropriate future time on another planet or in an after–life remain science fiction[54].
Nowadays, standard procedures for organ procurement and preservation require each organ to be flushed free of blood with a specially prepared ice–cold preservation solution, just prior to being removed from the donor. The organs are then placed in sterile containers, packaged in wet ice, and transported to the recipient's transplant center.
In order to allow inclusion of additional less–optimal donor categories, the organ procurement and initial perfusion technique are key factors toward an improved outcome in organ transplantation[55].
Good organ preservation starts with an effective blood washout of the donor organ. Major determinants to maintain graft viability, irrespective of the chosen preservation solution or method, are a rapid decrease in core temperature[56] and an equilibration between the intravascular preservation solution and the parenchyma[57]. Following the wash–out procedure, organ preservation is performed, using either static cold storage or continuous machine perfusion. Using simple cold storage methods, it is easy to transport organs for transplantation. However, despite the complexity and difficulty of organ transportation, continuous machine perfusion has some advantages by opening opportunities for viability assay and pretreatment of organs before transplantation.
Preservation solutions have been designed to ameliorate the adverse physiological and biochemical effects of ischemia under hypothermic conditions. Three principles are important in effective cold storage. First, the vascular wash–out during harvest should

rapidly cool the organs, remove the blood and allow balance between the cold storage solution and the tissue. Second, the cold storage solution should prevent cell swelling and interstitial edema formation by including substances that are osmotically active and impermeable to the cell. Impermeants and saccharides achieve homeostasis of the intracellular water content. Homeostasis of the interstitial compartments is achieved by counteracting a hydrostatic force during the initial wash–out using colloids. The intravascular fluid compartment does not need an effective component in static cold–storage. Third, the cold storage solutions should prevent excessive cellular acidosis by containing sufficient concentration of hydrogen–ion buffer, histidine or citrate[56,58].
Since its introduction by Belzer et al. in the late eighties, the University of Wisconsin (UW) solution has become the standard solution for the preservation of most organs in transplantation. Despite the fact that UW solution made extended cold preservation feasible, some studies have demonstrated that prolonged cold ischemic time of hepatic allografts enhance bacterial infection[59], cause biliary and hepatic artery complications[60,61] and increase the frequency of primary non function posttransplant[62].
The inclusion and importance of the colloid hydroxyethyl starch (HES) as one of the components of the UW solution has been both advocated and denied. HES prevents interstitial edema and has a beneficial effect on matrix metalo–proteinases[63] but at the price of a higher solution viscosity. Due to the presence of HES, the viscosity of UW solution at 4° C increased by a factor of 2.5 when compared with the viscosity of the same solution at 37° C[64].
"Folkert O. Belzer, the "father" of the University of Wisconsin preservation solution, was an outstanding practitioner of transplantation medicine, a brilliant, technically beautiful surgeon, and a superb educator–trainer of surgeons. However, his methods in the laboratory might be considered unorthodox. His goal was simple to improve organ preservation so he could offer his patients a better organ for a long and healthy life. In the early 1980s, Bob Hoffmann and I were trying to extend kidney preservation beyond 3 days; we believed that one of the problems was the lack of oxidizable substrates (fatty acids) in the perfusion fluid. We had trouble dissolving these fatty acids because we were no longer using serum albumin as a colloid but rather hydroxyethyl starch that did not bind fatty acids. One afternoon, Dr Belzer was drinking coffee and smoking his pipe in the laboratory, a pastime that consumed his off hours, led to great discussion between us, and catalyzed his mind. While reading the label on the coffee creamer carton, he noticed a list of monoglycerides and diglycerides and asked me about their purpose. I responded that these agents served as emulsifiers to help keep the lipid materials in solution. It was immediately obvious to me that the lights went on in his brain because he responded with, "Why not use this coffee creamer to solubilize fats in the perfusion solution?" I had no good explanation; hence, we tried the kidney perfusion with the addition of coffee creamer. (By the way, it did not work!) This is only one of the myriad "esoteric" compounds and chemicals we tried." *Southard JH.*[65]

1.3 Aim of research

The aim of this work was to investigate the efficiency of organ perfusion during acute organ support, as performed during extracorporeal mechanical blood circulation in cardiac patients, and during donor management, organ procurement and organ preservation prior to transplantation. The investigations were conducted in clinical studies, animal studies and in–vitro experimental settings. The efforts were concentrated on testing the diagnostic value of new, specific and sensitive biomarkers for organ injury, in order to help an early and effective therapeutic strategy. Given the complexity of this subject and the large diversity of clinical problems associated with impairment of organ perfusion, we approached only main points of debate in the current clinical world. The questions we addressed in the context of cardiopulmonary bypass associated morbidity concern the prophylactic use of corticosteroids, the isovolemic hemodilution, the therapeutic choice for plasma expenders, the myocardial protection and the corporeal temperature during extracorporeal circulation. In organ donation and transplantation, we investigated the consequences of cerebral injury in brain dead donors on organ viability. Impairment of perfusion during organ procurement and organ preservation was investigated in relation with the initial wash–out procedure. Beside clinical questions, the attention was also distributed towards the pathophysiologic mechanisms involved; vascular endothelial function and red blood cell function were especially addressed.

DEXAMETHASONE: BENEFIT AND PREJUDICE FOR PATIENTS UNDERGOING ON–PUMP CORONARY ARTERY BYPASS GRAFTING – A study on myocardial, pulmonary, renal, intestinal, and hepatic injury. (Chapter 2)

Administration of corticosteroids to patients undergoing on–pump cardiac surgery was demonstrated to inhibit the activation of the plasmatic and cellular inflammatory response, to decrease the pro– to anti–inflammatory interleukins ratio, to minimize tissue edema and to optimize the intravascular/extravascular fluid balance. Based on this constellation of findings, a routine prophylactic administration of corticosteroids was instituted in a multitude of clinical centers when performing on–pump heart surgery, assuming that inhibition of the systemic inflammatory response is automatically associated with clinical benefits. However, only a few clinical trials have been conducted to extend these results and to investigate the effect on clinical outcome in patients receiving corticosteroids.
As an original contribution to the issue of CPB related inflammatory response and organ injury, we document the effect of dexamethasone on perioperative myocardial, pulmonary, renal, intestinal and hepatic injury, as assessed by newly available specific and sensitive (bio)markers. Furthermore, to describe the effects of corticosteroids on the systemic inflammatory response, we measured cytokine response and systemic tryptase release as a marker of mast cells activation. Finally, a new hypothesis relating

tryptase to the attenuation of perioperative organ injury is discussed.

COMBINED STRATEGY TO LIMIT PERIOPERATIVE MYOCARDIAL, RENAL AND INTESTINAL TISSUE INJURY IN PATIENTS UNDERGOING ON–PUMP CORONARY ARTERY BYPASS GRAFTING (Chapter 3)

The available scientific data concerning the effectiveness and safety of cardiopulmonary bypass (CPB) for patients undergoing coronary artery bypass grafting (CABG) brings to attention several key principles to serve as basis for practical guidelines. Encouraged and generally accepted techniques are cold crystalloid cardioplegia, mild corporeal hypothermia and hemodilution to a hematocrit as low as 20%. Our therapeutic strategy aimed to explore, advance and optimize simultaneously more than one method of protection by eliminating several potential stress factors that might lead to injury and dysfunction during CPB: inadequate hypothermic cardioplegia, excessive hemodilution with subsequent need for perioperative blood transfusion, and corporeal hypothermia.
An experimental operative protocol was developed to meet multiple objectives: (1) homogeneous cooling of the myocardium by combining cold crystalloid cardioplegia technique with an additional intracavitary cooling of the heart; (2) prevention of excessive hemodilution by autologous priming of the extracorporeal circuit and partial recovery of the cardioplegic fluid; (3) corporeal normothermia.
The consequences on the postoperative organ injury and clinical outcome of the patients were investigated.

RED BLOOD CELL AGGREGATION DURING CARDIOPULMONARY BYPASS: A PATHOGENIC COFACTOR IN ENDOTHELIAL CELL ACTIVATION? (Chapter 4)

The relations between a low hematocrit and the adverse outcomes in patients undergoing CPB is extensively discussed in the literature[66]. There are also reports addressing the mechanical trauma of red blood cells[67] and the decrease in red blood cell deformability during extracorporeal circulation[68]. In our opinion, a complete chapter has been excluded from the discussion around the pathogenesis of the "post–CPB syndrome": modifications induced in red blood cell aggregation and potential consequences on microcirculation. The present study aimed to test the potential effect of priming solutions and the extracorporeal circulation on red blood cells aggregability and endothelial cell activation. We document the effects induced by two different prime solutions often used in the clinical practice, HAES–steril 6% and Voluven 6%. The clinical relevance and possible correlation between the pathophysiological mechanisms implicated are discussed.

ACUTE ISOVOLEMIC HEMODILUTION TRIGGERS PRO–INFLAMMATORY AND PRO–COAGULATORY ENDOTHELIAL ACTIVATION IN VITAL ORGANS: ROLE OF ERYTHROCYTES AGGREGATION (Chapter 5)

The essential role of erythrocytes as oxygen carriers is historically well established, however, their function to aggregate with consequences on homeostasis is strongly under debate. The aggregation property of red blood cells is mainly considered to be pathophysiologic, since aggregation is elevated in many disease states such as diabetes mellitus[69] and hypertension[70].
To date and rather remarkable, the scientific approach unravelling this subject has completely ignored the pathogenic potential of low erythrocyte aggregation states. Some authors have postulated the possibility that normal levels of aggregation may serve homeostasis, having functional significance for normal physiology, since red cell aggregation is normally present in humans and other "athletic" species[71,72]. This hypothesis, however, has never been investigated before, and also, never been placed in a clinical relevant context.
The pathogenicity of low erythrocyte aggregation could have major implications for hemodiluted patients. This situation routinely occurs in cardiac patients undergoing on–pump cardiopulmonary bypass who are severely hemodiluted due to therapeutic preoperative isovolemic hemodilution, priming of the extracorporeal circuit and large fluid infusions perioperatively. Excessive hemodilution prevails also during sustained fluid resuscitation in traumatic–hemorrhagic shock patients. In addition to the consequences of hypoxic stress, the implications of low erythrocyte aggregation during acute hemodilution might prove to be essential for a good understanding of microcirculation impairment and deteriorated tissue perfusion in these patients. Considering the sensitivity of vascular endothelium to variations in blood rheology, we hypothesized that low erythrocyte aggregation will be responsible for activation of vascular endothelium during acute isovolemic hemodilution.

CONSEQUENCES OF CEREBRAL INJURY IN BRAIN DEAD DONORS ON ORGAN VIABILITY: PROGRESS OF PRO–COAGULATORY AND PRO–INFLAMMATORY ENDOTHELIAL ACTIVATION (Chapter 6)

Endothelial activation in brain dead donors has gained lately considerably attention in the discussion concerning the pathology effects of brain death on donor organ quality prior to retrieval. In this respect, previous studies conducted in our laboratory suggest that an immune activation with increased endothelial cell activation and immediate early gene expression occurs after brain death induction[44]. Moreover, the expression of endothelial adhesion molecules (intercellular adhesion molecule–1 and vascular cell adhesion molecule–1) and the influx of leukocytes in the kidney occurs faster and is more profound when the hemodynamic instability in the brain dead donor is not

corrected[45]. In this way, the involvement of the activated vascular endothelium was linked incontestably to the progression of inflammation after brain death.
As an original contribution, this study aimed to analyze and document the time sequence for the progression of pro–inflammatory and pro–coagulatory endothelium activation, oxidative stress and organ viability in brain dead rat donors. Furthermore, this study investigated for the first time to our knowledge, extrahepatic fibrinogen synthesis in brain dead donors. We hypothesized that activated endothelium in the brain dead donor expresses and releases pro–inflammatory and pro–coagulatory factors into the circulation that will mediate inflammation, platelet adhesion and possibly promote microthrombosis. In addition, we expected that due to activation of endothelium and hypoxic stress, the oxidative stress and brain death related organ dysfunction are enhanced.
This investigation could pinpoint the time points necessary for anti–inflammatory and anti–coagulatory therapeutic interventions, that could effectively reduce endothelial activation, prevent platelet adhesion and leukocyte infiltration, and possibly slow down an ongoing organ deterioration during brain death.

HYPERAGGREGATING EFFECT OF HYDROXYETHYL STARCH COMPONENTS AND UNIVERSITY OF WISCONSIN SOLUTION ON HUMAN RED BLOOD CELLS: A RISK OF IMPAIRED GRAFT PERFUSION IN ORGAN PROCUREMENT? (Chapter 7)

The viability of organ grafts depends on several factors such as cold ischemia time, the perfusion procedure, preservation methods and reperfusion quality. The efficacy of perfusion during the initial wash–out procedure, however, has to be also considered a major determinant of functional recovery after transplantation[55,73].
Preservation solutions have been designed to ameliorate the adverse physiological and biochemical effects of ischemia under hypothermic conditions. The inclusion and importance of the colloid hydroxyethyl starch (HES) as one of the components of the University of Wisconsin (UW) solution has been both advocated and denied. In an experimental setting analyzing the effect of HES on the rheological properties of blood, Corry and collaborators have drawn the attention to the aggregating effect of HES on erythrocytes[74].
The present study aimed to test the effect of HES and UW solution on red blood cell aggregability and to correlate aggregation parameters with HES molecular weight. In addition, the study aimed to detail the extent and kinetics of HES–induced human RBC aggregation, as well as the morphological characterization of these aggregates. It could be possible that by identifying the RBC hyperaggregating effect of UW solution as an etiology–related factor for these complications immediate function, patient and graft survival would improve.

References

1. Reilly PM, Wilkins KB, Fuh KC, Haglund U, Bulkley GB. The mesenteric hemodynamic response to circulatory shock: an overview. *Shock.* 2001;15:329–43.

2. Fowler RA, Lapinsky SE, Hallett D, Detsky AS, Sibbald WJ, Slutsky AS, Stewart TE. Toronto SARS Critical Care Group. Critically ill patients with severe acute respiratory syndrome. *JAMA.* 2003;290:367–73.

3. Habib RH, Zacharias A, Schwann TA, Riordan CJ, Durham SJ, Shah A. Adverse effects of low hematocrit during cardiopulmonary bypass in the adult: should current practice be changed? *J. Thorac. Cardiovasc. Surg.* 2003;125:1438–50.

4. Girardi LN, Krieger KH, Altorki NK, Mack CA, Lee LY, Isom OW. Ruptured descending and thoracoabdominal aortic aneurysms. *Ann. Thorac. Surg.* 2002;74:1066–70.

5. Eltzschig HK, Collard CD. Vascular ischaemia and reperfusion injury. *Br. Med. Bull.* 2004;70:71–86.

6. Ruokonen E, Parviainen I, Uusaro A. Treatment of impaired perfusion in septic shock. *Ann. Med.* 2002;34:590–7.

7. Beck GCh, Brinkkoetter P, Hanusch C, Schulte J, van Ackern K, van der Woude FJ,Yard BA. Clinical review: immunomodulatory effects of dopamine in general inflammation. *Crit. Care.* 2004;8:485–91.

8. Stochetti N, Furlan A, Volta F. Hypoxemia and arterial hypotension at the accident scene in head injury. *J. Trauma.* 1996;40:764–767.

9. Holtzer S, Vigue B, Ract C, Samii K, Escourrou P. Hypoxia–hypotension decreases pressor responsiveness to exogenous catecholamines after severe traumatic brain injury in rats. *Crit. Care Med.* 2001;29:1609–14.

10. Fou AA and Gibbon HJ: the first 20 years of the heart-lung machine. *Tex. Heart Inst. J.* 1997;24:1–8.

11. JH Gibbon, Jr, Application of a mechanical heart and lung apparatus to cardiac surgery. *Minn. Med.* 1954;37:171–185.

12. JW Kirklin, Open heart surgery at the Mayo Clinicthe 25th anniversary. *Mayo. Clinic. Proc.* 1980;50:339–341.

13. The American Heritage® Dictionary of the English Language, Fourth Edition. Copyright © 2000 by Houghton Mifflin Company. Published by the Houghton Mifflin Company.

14. Birnbaum DE. Extracorporeal circulation in non–cardiac surgery. *Eur. J. Cardiothorac. Surg.* 2004;26:S82–5.

15. Goldman AP, Cassidy J, de Leval M, Haynes S, Brown K, Whitmore P, Cohen G, Tsang V, Elliott M, Davison A, Hamilton L, Bolton D, Wray J, Hasan A, Radley-Smith R, Macrae D, Smith J. The waiting game: bridging to paediatric heart transplantation. *Lancet.* 2003;362:1967–70.

16. Jansen NJ, van Oeveren W, van den Broek L, Oudemans–van Straaten HM, Stoutenbeek CP, Joen MC et al. Inhibition by dexamethasone of the reperfusion phenomena in cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg.* 1991;102:515–25.

17. Chaney MA. Corticosteroids and cardiopulmonary bypass: a review of clinical investigations. *Chest.* 2002;121:921–31.

18. Paparella D, Yau TM, Young E. Cardiopulmonary bypass induced inflammation: pathophysiology and treatment. An update. *Eur. J. Cardiothorac. Surg.* 2002;21:232–44.

19. El Azab SR, Rosseel PM, de Lange JJ, et al. Dexamethasone decreases the pro– to anti–inflammatory cytokine ratio during cardiac surgery. *Br. J. Anaesth.* 2002;88:496–501.

20. von Spiegel T, Giannaris S, Wietasch GJ, et al. Effects of dexamethasone on intravascular and extravascular fluid balance in patients undergoing coronary bypass surgery with cardiopulmonary bypass. *Anesthesiology.* 2002;96:827–34.

21. van Oeveren W. Biomaterials for rotary blood pumps. *Artif. Organs.* 1995;19:603–7.

22. de Vries AJ, Gu YJ, Post WJ, Vos P, Stokroos I, Lip H, van Oeveren W. Leucocyte depletion during cardiac surgery: a comparison of different filtration strategies. *Perfusion.* 2003;18:31-8.

23. van Oeveren W, Kazatchkine MD, Descamps-Latscha B, Maillet F, Fischer E, Carpentier A, Wildevuur CR. Deleterious effects of cardiopulmonary bypass. A prospective study of bubble versus membrane oxygenation. *J. Thorac. Cardiovasc. Surg.* 1985;89:888–99.

24. Ashraf S, Butler J, Tian Y, Cowan D, Lintin S, Saunders NR, Watterson KG, Martin PG. Inflammatory mediators in adults undergoing cardiopulmonary bypass: comparison of centrifugal and roller pumps. *Ann. Thorac. Surg.* 1998;65:480–4.

25. Cooley DA, Frazier OH. The past 50 years of cardiovascular surgery. *Circulation.* 2000;102:IV87–IV93.

26. Svenmarker S, Sandstrom E, Karlsson T, Jansson E, Haggmark S, Lindholm R, Appelblad M, Aberg T. Clinical effects of the heparin coated surface in cardiopulmonary bypass. *Eur. J. Cardiothorac. Surg.* 1997;11:957–64.

27. de Vroege R, van Oeveren W, van Klarenbosch J, Stooker W, Huybregts MA, Hack CE, van Barneveld L, Eijsman L, Wildevuur CR. The impact of heparin–coated cardiopulmonary bypass circuits on pulmonary function and the release of inflammatory mediators. *Anesth. Analg.* 2004;98:1586–94.

28. van Oeveren W, Jansen NJ, Bidstrup BP, Royston D, Westaby S, Neuhof H, Wildevuur CR. Effects of aprotinin on hemostatic mechanisms during cardiopulmonary bypass. *Ann. Thorac. Surg.* 1987;44:640–5.

29. Tabuchi N, De Haan J, Boonstra PW, Huet RC, van Oeveren W. Aprotinin effect on platelet function and clotting during cardiopulmonary bypass. *Eur. J. Cardiothorac. Surg.* 1994;8:87–90.

30. Mojcik CF, Levy JH. Aprotinin and the systemic inflammatory response after cardiopulmonary bypass. *Ann. Thorac. Surg.* 2001;71:745–754.

31. Paparella D, Yau TM, Young E. Cardiopulmonary bypass induced inflammation: Pathophysiology and treatment. An update. *Eur. J. Cardiothorac. Surg.* 2002;21:232–244.

32. Menasche P. The systemic factor: The comparative roles of cardiopulmonary bypass and off–pump surgery in the genesis of patient injury during and following cardiac surgery. *Ann. Thorac. Surg.* 2001;72:S2260–S2265.

33. Morariu AM, Loef BG, Rietman GW, Aarts LPHJ, Rakhorst G, van Oeveren W, Epema AH. Dexamethasone: benefit and prejudice for patients undergoing on–pump coronary artery bypass grafting. A study on myocardial, pulmonary, renal, intestinal, and hepatic injury. *Chest.* 2005

34. Deiwick M, Hoffmeier A, Tjan TD, Krasemann T, Schmid C, Scheld HH. Heart failure in children – mechanical assistance. *Thorac. Cardiovasc. Surg.* 2005;53:S135–40.

35. Cabrer C, Manyalich M, Valero R, Garca-Fages LC. Timing used in the different phases of the organ procurement process. *Transplant Proc.* 1992;24:22–23.

36. Power BM, Van Heerden PV. The physiologic changes associated with brain death – current concepts and implications for the treatment of the brain dead organ donor. *Anaesth. Intens. Care.* 1995;23–26.

37. Novitzky D, Cooper DKC, Reichart B. Hemodynamic and metabolic responses to hormonal therapy in brain–dead potential organ donors. *Transplantation.* 1987;43:852–54.

38. Pinelli G, Mertes P-M, Carteaux J-P, et al. Myocardial effects of experimental acute brain death: evaluation by hemodynamic and biological studies. *Ann. Thorac. Surg.* 1995;60:1729–34.

39. Bittner HB, Chen EP, Kendall SW, Van Trigt P. Brain death alters cardiopulmonary hemodynamics and impairs right ventricular power reserve against an elevation of pulmonary vascular resistance. *Chest.* 1997;111:706–11.

40. Gramm HJ, Meinhold H, Bickel U, Zimmermann J, von Hammerstein B, Keller F, Dennhardt R, Voigt K. Acute endocrine failure after brain death? *Transplantation.* 1992;54:851–857.

41. Riou B, Kalfon P, Arock M, Goarin JP, Saada M, Viars P. Cardiovascular consequences of severe hyphosphataemia in brain–dead patients. *Br. J. Anaesth.* 1995;74:424–429.

42. Takada M, Nadeau KC, Hancock WW, Mackenzie HS, Shaw GD, Waaga AM, Chandraker A, Sayegh MH, Tilney NL. Effects of explosive brain death on cytokine activation of peripheral organs in the rat. *Transplantation.* 1998;65:1533–1542.

43. Zhai QH, Futrell N, Chen FJ. Gene expression of IL–10 in relationship to TNF–alpha, IL–1beta and IL–2 in the rat brain following middle cerebral artery occlusion. *J. Neurol. Sci.* 1997;152:119–124.

44. van der Hoeven JA, Ploeg RJ, Postema F, Molema I, de Vos P, Girbes AR, van Suylichem PT, van Schilfgaarde R, Ter Horst GJ. Induction of organ dysfunction and up–regulation of inflammatory markers in the liver and kidneys of hypotensive brain dead rats: a model to study marginal organ donors. *Transplantation.* 1999;68:1884–90.

45. van der Hoeven JA, Molema G, Ter Horst GJ, Freund RL, Wiersema J, van Schilfgaarde R, Leuvenink HG, Ploeg RJ. Relationship between duration of brain death and hemodynamic (in)stability on progressive dysfunction and increased immunologic activation of donor kidneys. *Kidney Int.* 2003;64:1874–82.

46. Mackersie RC, Bronsther LO, Shackford SR. Organ procurement in patients with fatal head injuries. *Ann. Surg.* 1991;213:143–150.

47. Darby JM, Stein K, Grenvik A, Stuart S. Approach to management of the heartbeating brain dead organ donor. *JAMA.* 1989;261: 2222–2228.

48. Wheeldon DR, Potter CDO, Oduro A, Wallwork J, Large SR. Transforming the "unacceptable" donor: outcomes from the adoption of a standardized donor management technique. *J. Heart Lung Transplant.* 1995;14:734–742.

49. Zaroff JG, Rosengard BR, Armstrong WF et al. ASTS–AST Consensus Conference Report. Maximizing Use of Organs Recovered from the Cadaver Donor: Cardiac Recommendations. Crystal City, Virginia, USA: *ASTS-AST*; 2001;2829.

50. Rosendale JD, Chabalewski FL, McBride MA, Garrity ER, Rosengard BR, Delmonico FL, Kauffman HM. Increased transplanted organs from the use of a standardized donor managementprotocol. *Am. J. Transplant.* 2002;2:761–8.

51. Holmquist M, Chabalewski F, Blount T, Edwards C, McBride V, Pietroski R. A critical pathway: guiding care for organ donors. *Crit. Care Nurse.* 1999;19:8498.

52. Rosendale JD, Kauffman HM, McBride MA, Chabalewski FL, Zaroff JG, Garrity ER, Delmonico FL, Rosengard BR. Aggressive pharmacologic donor management results in more transplanted organs. *Transplantation.* 2003;75:482–7.

53. Sekela ME, Young JB, Short HD, Whisennand HH, Noon GP. Multi–organ procurement utilizing cardiopulmonary bypass and profound hypothermic circulatory arrest. *Clin. Transplant.* 1991;5:318–21.

54. Marshall VC, Jablonski P, Scott DF and Howden BO. Organ and Tissue Preservation for Transplantation. P J Morris and R A Malt (eds) Oxford Textbook of Surgery Oxford University Press, Oxford, 1994;651–662.

55. 't Hart NA, van der Plaats A, Leuvenink HG, Wiersema-Buist J, Olinga P, van Luyn MJ, Verkerke GJ, Rakhorst G, Ploeg RJ. Initial blood washout during organ procurement determines liver injury and function after preservation and reperfusion. *Am. J. Transplant.* 2004;4:1836–44.

56. Ploeg RJ, Goossens D, McAnulty JF, Southard JH, Belzer FO. Succesfull 72–hour cold storage of dog kidneys with UW solution. *Transplantation.* 1988;46:191196.

57. Yamauchi JI, Richter S, Vollmar B, Menger MD, Minor T. Warm preflush with streptokinase improves microvascular procurement and tissue integrity in liver graft retrieval from non–heart–beating donors. *Transplantation.* 2000;69:1780-4.

58. 't Hart NA, Leuvenink HGD, Ploeg RJ. New solutions in organ preservation. *Transplantation Reviews.* 2002;16:131–141.

59. Nuno J, Cuervas-Mons V, Vicente E, Turrion V, Pereira F, Mora NP, et al. Prolonged graft cold ischemia: a risk factor for early bacterial and fungal infection in liver transplant recipient. *Transplantation Proc.* 1995;27:2323–5.

60. Carrasco L, Sanchez-Bueno F, Sola J et al. Effects of cold ischemia time on the graft after orthotopic liver transplantation. *Transplantation.* 1996;61:393–6.

61. Mor E, Schwartz ME, Sheiner PA et al. Prolonged preservation in University of Wisconsin solution associated with hepatic artery thrombosis after orthotopic liver transplantation. *Transplantation.* 1993;56:1399–402.

62. Adam R, Bismuth H, Diamond T et al. Effect of extended cold ischemia with UW solution on graft function after liver transplantation. *Lancet.* 1992;340:1373–7.

63. Ploeg RJ, van Bockel JH, Langendijk PT et al. Effect of preservation solution on results of cadaveric kidney transplantation. The European Multicentre Study Group. *The Lancet.* 1992;340:129–37.

64. Tojimbara T, Wicomb WN, Garcia-Kennedy R, Burns W, Hayashi M, Collins G, Esquivel CO. Liver transplantation from non-heart beating donors in rats: influence of viscosity and temperature of initial flushing solutions on graft function. *Liver Transpl. Surg.* 1997;3:39–45.

65. Southard JH. Coffee creamer, the bionic man, and organ preservation. *Surgery.* 2002;131:228–9.

66. Groom RC. High or low hematocrits during cardiopulmonary bypass for patients undergoing coronary artery bypass graft surgery? An evidence–based approach to the question. *Perfusion.* 2002;17:99–102.

67. Svenmarker S, Jansson E, Stenlund H, Engstrom KG. Red blood cell trauma during cardiopulmonary bypass: narrow pore filterability versus free haemoglobin. *Perfusion.* 2000;15:33–40.

68. Kameneva MV, Undar A, Antaki JF, Watach MJ, Calhoon JH, Borovetz HS. Decrease in red blood cell deformability caused by hypothermia, hemodilution, and mechanical stress: factors related to cardiopulmonary bypass. *ASAIO J* 1999;45:307–10.

69. Martinez M, Vaya A, Server R, Gilsanz A, Aznar J. Alterations in erythrocyte aggregability in diabetics: the influence of plasmatic fibrinogen and phospholipids of the red blood cell membrane. *Clin. Hemorheol. Microcirc.* 1998;18:253–8.

70. Cicco G, Pirrelli A. Red blood cell (RBC) deformability, RBC aggregability and tissue oxygenation in hypertension. *Clin. Hemorheol. Microcirc.* 1999;21:169–77.

71. Popel AS, Johnson PC, Kameneva MV, Wild MA. Capacity for red blood cell aggregation is higher in athletic mammalian species than in sedentary species. *J. Appl. Physiol.* 1994;77:1790–4.

72. Bishop JJ, Nance PR, Popel AS, Intaglietta M, Johnson PC. Effect of erythrocyte aggregation on velocity profiles in venules. *Am. J. Physiol. Heart Circ. Physiol.* 2001;280:222–36.

73. Ploeg RJ , D'Alessandro AM, Knechtle SJ et al. Risk factors for primary dysfunction after liver transplantation – a multivariate analysis. *Transplantation.* 1993;55:807–13.

74. Corry WD, Jackson LJ, Seaman GV. Action of hydroxyethyl starch on the flow properties of human erythrocyte suspensions. *Biorheology.* 1983;20:705–17.

Chapter 2

Organ Perfusion During Cardiopulmonary Bypass: Therapeutic Effect of Dexamethasone.

Dexamethasone: Benefit and Prejudice for Patients Undergoing On–pump Coronary Artery Bypass Grafting

A study on myocardial, pulmonary, renal, intestinal, and hepatic injury.

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Abstract

Study Objectives

Cardiac surgery with cardiopulmonary bypass (CPB) results in perioperative organ damage caused by the systemic inflammatory response syndrome (SIRS) and ischemia/reperfusion injury. Administration of corticosteroids before CPB has been demonstrated to inhibit the activation of the systemic inflammatory response. However, the clinical benefits of corticosteroid therapy are controversial. This study was designed to document the effects of dexamethasone on cytokine release and perioperative myocardial, pulmonary, renal, intestinal and hepatic damage, as assessed by specific and sensitive (bio)markers.

Design and Patients

A prospective, double–blind, placebo–controlled, randomized trial for dexamethasone was conducted in 20 patients, receiving either dexamethasone (1 mg/kg before anesthesia induction and 0.5 mg/kg after 8 hours) (n=10) or placebo (n=10). Different markers were used to asses the SIRS: Interleukin–6 (IL–6), Interleukin–8 (IL–8), Interleukin–10 (IL–10), C–reactive protein (CRP), tryptase, and organ injury: heart (plasma heart–type fatty acid binding protein H–FABP, Troponin I, Creatine kinase MB, CK–MB), kidneys (N–acetyl–glucosaminidase, NAG, microalbuminuria), intestine (intestine/liver–type FABP, I/L–FABP), liver (αGlutathione S–transferase, αGST).

Results

Dexamethasone modulated the SIRS with lower pro–inflammatory (IL–6,8) and higher anti–inflammatory (IL–10) interleukin levels. CRP and tryptase were lower in the dexamethasone group. Cardiac Troponine I values were lower in the dexamethasone–group at 6 h ICU (p=0.009). Patients in dexamethasone group had longer time to tracheal extubation (18.86±1.13 h versus 15.01±0.99 h, p=0.02) with lower oxygenation index at that time (PaO_2/FiO_2 ratios: 37.17±1.8 kPa versus 29.95±2.1 kPa, p=0.009). Postoperative glucose (10.7±0.6 mmol/L versus 7.4±0.5 mmol/L, p=0.005) was higher in the dexamethasone group. Serum glucose was independently associated with intestinal injury (urine I–FABP peak: R^2=42.5%, B=114.4±31.4, Sig.=0.002, urine L–FABP peak: R^2=47.3%, B=7714.1±1920.9, Sig.=0.001) and renal injury (urine NAG: R^2=32.1%, B=0.21±0.07, Sig.=0.009). Tryptase peaks correlated negatively with peaks of intestinal and renal injury (bio)markers.

Conclusions

Even while inhibiting SIRS, dexamethasone treatment offered no protection against transient, subclinical, perioperative abdominal organ damage. Tryptase release could have a preconditioning effect, offering protection against perioperative intestinal and renal damage. Dexamethasone treatment resulted in more pronounced postoperative pulmonary dysfunction, prolonged time to tracheal extubation and initiated postoperative hyperglycaemia in patients undergoing elective on–pump CABG.

2.1 Introduction

Organ damage after cardiac surgery with cardiopulmonary bypass (CPB) is caused by two related pathophysiological mechanisms: the systemic inflammatory response syndrome (SIRS) and ischemia/reperfusion injury.
SIRS is triggered by the exposure of blood to large areas of synthetic materials of the extracorporeal circuit. It causes a complex inflammatory reaction involving activation of complement, platelets, neutrophils, monocytes and macrophages with increased blood concentrations of cytokines and leukotriens. Additionally, SIRS initiates activation of the coagulation, fibrinolytic and kallikrein cascades. A subsequent increase in endothelial cell permeability allows transvascular migration of activated leukocytes into the tissues with additional vascular and parenchymal damage[1,2].
The ischemia/reperfusion injury is triggered mainly in heart and lungs secondary to aortic cross–clamping and cardioplegic arrest[3,4]. During aorta cross clamping the heart is excluded from the circulation, being protected by cardioplegia and hypothermia. The lungs are deprived as well of pulmonary blood flow. Ischemia/reperfusion injury has been documented also in other organs such as kidneys and intestine, probably due to alterations in blood flow at the microcirculatory level[5,6]. Preoperative administration of corticosteroids to patients undergoing cardiac surgery with CPB has been demonstrated to inhibit the activation of the plasmatic and cellular inflammatory response[7], to decrease the pro– to anti–inflammatory interleukins ratio[8], and to minimize tissue edema[9]. Based on these findings corticosteroids are routinely used in a considerable number of institutions. The studies on the clinical benefits, however, show conflicting results when referring to changes in hemodynamic, pulmonary function and glucose metabolism[10–13]. Recent clinical investigations by Chaney et al.[12,13] indicated that methylprednisolone offers no clinical benefit, and may in fact be detrimental by initiating postoperative hyperglycemia and delaying postoperative tracheal extubation for undetermined reasons.
As an original contribution to the issue of CPB related SIRS and organ injury, we document the effect of dexamethasone on perioperative myocardial, pulmonary, renal, intestinal and hepatic damage, as assessed by newly available specific and sensitive (bio)markers. Furthermore, to describe the effects of corticosteroids on the systemic inflammatory response, we measured cytokine response and systemic tryptase release as a marker of mast cells activation[14]. Finally, a new hypothesis relating tryptase to the attenuation of perioperative organ injury will be discussed.

2.2 Patients, Materials and Methods

Patients

The study was designed as a prospective, double blind, placebo–controlled, randomized trial for dexamethasone. After approval by the hospital ethics committee and

written informed consent, patients scheduled for first time coronary artery revascularization were studied. All patients included in the study had coronary artery disease with normal renal function (as assessed by a serum creatinine of less than 120 μmol.liter^{-1}), normal hepatic, cerebral and cardiac function (ejection fraction > 45%). Patients with diabetes, recent myocardial infarction, unstable angina, or recent use of radiocontrast agents and corticosteroids were excluded, as these conditions might be associated with increased baseline levels of the markers used in this study.

Anesthetic management

Patients (n=20) were randomized in a double–blinded fashion to receive either dexamethasone or placebo. A baseline serum glucose sample was obtained after overnight fasting. In the treatment group patients received dexamethasone 1 mg.kg^{-1} at induction of anesthesia and 0.5 mg.kg^{-1} 8 hours later. Patients in the control group received a placebo at the same time points. Anesthesia was provided according to a fixed protocol[15]. Premedication consisted of oral diazepam 10–15 mg 2 hours preoperatively. After insertion of peripheral venous and radial cannulae under local analgesia, general anesthesia was induced with sufentanil (2.5 μg.kg^{-1}) and midazolam (0.1 mg.kg^{-1}). Tracheal intubation was achieved with pancuronium (0.1 mg.kg^{-1}) and the lungs were ventilated with air and oxygen (FiO_2=0.4). A flow–directed pulmonary artery catheter was inserted into the right internal jugular vein, and an indwelling bladder catheter was used for urine collection. Anesthesia was maintained with sufentanil, midazolam, and pancuronium. Cefuroxim (1500 mg) was administered after induction. Hydroxyethyl starch 200/0.5 6% solution and lactated Ringers solution were used to obtain a mean arterial pressure (MAP) > 60 mmHg, to maintain filling pressures and cardiac output. Transfusion of packed cells were given at a hemoglobin < 4.5 mmol.L^{-1}. According to standard care in our clinic, intravenous insulin was started at a serum glucose > 10 mmol.L^{-1}. Inotropic support with dopamine was started at a cardiac index < 2.2 L.min.m^{-2}. Diuretics, mannitol or aprotinin were not administered during the entire study period. Patient characteristics and perioperative variables were recorded prospectively.

Cardiopulmonary Bypass

Non–pulsatile CPB was performed using a roller pump (CAPS HLM, Stökert Instruments, Germany) and a membrane oxygenator (Cobe Optima; Cobe Laboratories; Lakewood, CO). The extracorporeal circuit was primed with 500 ml HES 200/0.5 6% and 1000 ml lactated Ringers solution. During CPB, the flow was maintained at 2.4 L.min.m^{-2} with moderate hypothermia (32° C) and α–stat regulation of blood pH. Cold St. Thomas solution was infused into the aortic root to maintain cardioplegia during aortic cross–clamping. During CPB, the mean arterial pressure was allowed to vary between 60 and 90 mmHg. Deviations were corrected with phenylephrine or

nitroglycerine. The urine collection was divided in six intervals: (1) preoperative (baseline): during 12 hours prior to surgery, (2) preheparinization: from skin incision to systemic heparinization, (3) sternum closure: from heparinization to sternum closure, (4) 2 h ICU: during 2 hours postoperative, (5) 6 h ICU: 2 to 6 hours postoperative, (6) 24 h ICU: 6 h to 24 h postoperative. Urinary excretion of the measured biomarkers was calculated as ratio to urine creatinine concentration and adjusted to time interval in order to correct for changes in urinary flow:

[Urinary production = measured urine concentration / (time interval for urine collection × urinary creatinine concentration)]

Blood sampling was performed before induction of anesthesia (preinduction), 5 minutes after Aortic cross clamp release (Ao clamp release), 6 h postoperative (6 h ICU), and 24 h postoperative (24 h ICU). Urine and plasma were stored at –20° C until assay.

(Bio)markers

Inflammatory biomarkers

- Interleukin–6 (IL–6), Interleukin–8 (IL–8), Interleukin–10 (IL–10) – solid–phase, enzyme–labelled, chemiluminescent sequential immunometric assay (IMMULITE, EURO/DPC Ltd, USA)
- *C Reactive protein* – high sensitive ELISA (HaemoScan, Groningen, The Netherlands).
- *Tryptase* (proteolytic trypsin–like enzyme released from activated mast cells) – enzymatic assay (HaemoScan, Groningen, The Netherlands).
- *Serum glucose* concentration was determined using a Vitros analyzer (Ortho Clinical Diagnostics; Beerse, Belgium).

Myocardial injury biomarkers

- *plasma heart–type fatty acid binding protein* (H–FABP–cyrosolic protein released from injured myocytes) – ELISA kit (HyCult Biotechnology B.V., Uden, The Netherlands).
- *cardiac Troponin I* (cTnI–myofibrillar protein released from injured myocytes) – microparticle enzyme immunoassay (AxSYM, ABBOT Laboratories, USA).
- *Creatine kinase MB (CK–MB)* activity – Vitros analyzer (Ortho Clinical Diagnostics; Beerse, Belgium).

Kidney injury biomarker

- *Urine N–acetyl–glucosaminidase* (NAG–enzyme released from injured proximal renal tubules) – modified enzyme assay according to Lockwood[16] at pH 4.5 and corrected for non–specific conversion (HaemoScan, Groningen, The Netherlands).

Intestinal injury biomarkers

- *Intestinal/Liver–type fatty acid binding proteins* (I/L–FABP–cytosolic proteins in the enterocytes released into the blood stream and excreted by kidney early in the course of intestinal ischemia[17]) ELISA kit (HyCult Biotechnology BV, Uden, The Netherlands).

Hepatic injury biomarkers

- α*Glutathione S–transferase* (αGST – enzyme released from centrolobular and periportal damaged hepatocytes reported as having uniform hepatic distribution, high cytosol concentration, and short half–life[18])– enzyme immunoassay (Biotrin International Ltd., Dublin, Ireland).

Statistical Analysis

The statistical analysis was performed using SPSS (Statistical Package for the Social Sciences). A power analysis, based on previous studies of IL–6 and IL–8 plasma levels in this population suggested that at least 20 patients have to be studied in order to detect a 1 SD difference between the two groups, with a reliability of 5% and a power of 80%. Before analysis, the data was tested for distribution according to Kolmogorov–Smirnov goodness of fit test. The variation of the urinary and plasma markers over the study period and the differences between groups were investigated using repeated measures ANOVA. A total area under curve (AUC) was calculated for all plasma biomarkers. Continuous variables were compared by means of parametric (Student T Test) or nonparametric tests (Mann–Whitney). Fishers exact test was used to compare discrete variables. Correlation analysis between variables was tested using Spearman correlation test. Regression analysis was used to detect predictors for organ injury. Statistical significance was accepted at $p<0.05$. Results are presented as mean±SEM (unless stated otherwise).

2.3 Results

All twenty patients included completed the study and survived the hospital stay. The following complications were observed: revision for bleeding (n=2); perioperative myocardial infarction (n=1); atrial fibrillation (n=1); nosocomial pneumonia (n=1). Seven patients in the dexamethasone group and two patients in the placebo group (Fischer's exact test p=0.025) received dopamine less than $5\,\mu g.kg^{-1}.min^{-1}$ because of low cardiac index. Six patients in the dexamethasone group and one patient in

the placebo group received insulin to regulate serum glucose in the postoperative period (Fischer's exact test p=0.057). Fever (highest measured rectal temperature) was more prominent in the placebo group during the first 24 hours postoperatively. Additional patients' characteristics and operative data are shown in Table 2.1. The patients in the dexamethasone group were slightly older than the patients in placebo group. However, the age did not prove to be a predictor for any of the biomarkers tested. Marked blood loss occurred in one patient in the dexamethasone group, who received 13 units of blood more than 6 h after bypass. As this would affect only the last time point of the study, this patient was included in the analysis.

	Placebo group Mean±SEM	Dexamethasone group Mean±SEM	P value
No. of patients	10	10	/
Male/Female	9/1	8/2	/
Age 95%CI (mean) (y)	53.4-65.5 (59.5)	63.4-72.1 (67.8)	0.02
Body surface area (m^2)	1.96±0.07	1.92±0.03	0.58
Perfusion time (min)	95.9±7.6	115.7±8.8	0.10
Aorta clamping time(min)	61.7±5.7	74.8±6.9	0.16
MAP Preoperative	90.4±5.6	96.1±4.8	0.52
MAP ICU admission	73.7±4.8	74.2±4.4	0.79
MAP 24h ICU (mmHg)	80.9±3.4	93.8±4.5	0.053
SVRI Preoperative	2263±212	2429±251	0.85
SVRI ICU admission	2134±283	1889±181	0.57
SVRI 24h ICU ($dynes/cm^5/m^2$)	1630±151	2030±239	0.35
CI Preoperative	2.6±0.2	2.6±0.4	0.79
CI ICU admission	2.8±0.3	2.8±0.3	0.63
CI 24h ICU ($L/min/m^2$)	3.7±0.3	3.6±0.3	0.96
preoperative PaO_2/FiO_2	52.68±4.91	51.65±4.54	0.63
extubation PaO_2/FiO_2 (kPa)	37.17±1.8	29.95±2.1	0.009
Intubation time (h)	15.01±0.99	18.86±1.13	0.02
Highest measured rectal temperatures (° C)	37.9±0.8	37.2±0.3	0.02
Units of blood transfused	2.2±3.6	1.3±1.6	0.51

Table 2.1: Patient characteristics and operative data (mean ± standard error of the mean). MAP = Mean arterial pressure, SVRI = systemic vascular resistance index, CI = cardiac index, PaO_2/FiO_2 = oxygen index. Statistics: Continuous variables where compared by means of parametric (Student T Test) or nonparametric tests (Mann–Whitney). Fisher's exact test was used to compare discrete variables.

Inflammatory biomarkers

Plasma levels of pro–inflammatory cytokines IL–6 and IL–8 increased significantly (Wilks Sig.<0.001) in both groups, with a lower response in the dexamethasone group (lower total AUC in dexamethasone group for both IL–6 and IL–8, p<0.001). The peak values were measured at 6 h ICU for IL–6, and during the sternum closure for IL–8 (Fig. 2.1a,b). The IL–6 values were significantly lower in the dexamethasone group at 6 h and 24 h ICU (p<0.001). IL–8 was significantly lower in the dexamethasone group after aortic clamp release (p=0.023), during sternum closure (p<0.001), at 6h ICU (p=0.003), and total AUC (p<0.001).
IL–6 values at 24 h ICU were higher than baseline values in both groups (p<0.001). IL–8 values returned to baseline values after 24 hours in both groups.
Plasma IL–10 increased significantly (Wilks Sig.<0.001) in both groups. In the dexamethasone group plasma IL–10 had a ≈4 fold higher peak at sternum closure. The differences between groups were statistically significant after aortic clamp release and sternum closure (p<0.001), 6 h ICU (p=0.029), and total AUC (p<0.001) (Fig. 2.1c). The IL–10 values returned to baseline values after 6 h ICU in both groups.
Plasma levels of CRP did not increase during the operation. The differences between groups on their overall plasma CRP were statistically significant (p=0.048). The dexamethasone group had lower total AUC (p=0.028), and lower CRP levels at 6 h ICU (4.9±1 μg/ml in dexamethasone group versus 39.5±24.9 μg/ml in the placebo group, p=0.043), at 24 h ICU (842.7±524 μg/ml in dexamethasone group versus 2463.5±968 μg/ml in the placebo group, p=0.028).
Tryptase increased significantly during operation in both groups (Wilks Sig.=0.018) (Fig. 2.1d). In the dexamethasone group, tryptase concentrations increased only moderately with peak values at sternum closure. In the placebo group, the values rose abruptly reaching peak values immediately after releasing the aortic cross–clamp, and decreased after sternum closure. The tryptase values were significantly lower in the dexamethasone group after aortic clamp release (p=0.015), during sternum closure (p=0.009), and total AUC (p=0.05). Tryptase values returned to baseline values after 6 h ICU in both groups.

Myocardial injury biomarkers

The release patterns of the myocardial damage markers had a different time course. Plasma H–FABP (Fig. 2.2a) started to rise directly after aortic cross clamp release, reaching peak values after 1.23 hours (95%CI=0–2.66 h), which was significantly earlier (p<0.001) than the peak values of cTnI and CK–MB (cTnI: mean=14.1 h, 95%CI=6.36–21.84 h; CK–MB: mean=16.35, 95%CI=9.23–23.47 h). The only difference between the treatment groups, was observed at 6 h ICU, with a lower value of cTnI in the dexamethasone group (p=0.009) (Fig. 2.2b).

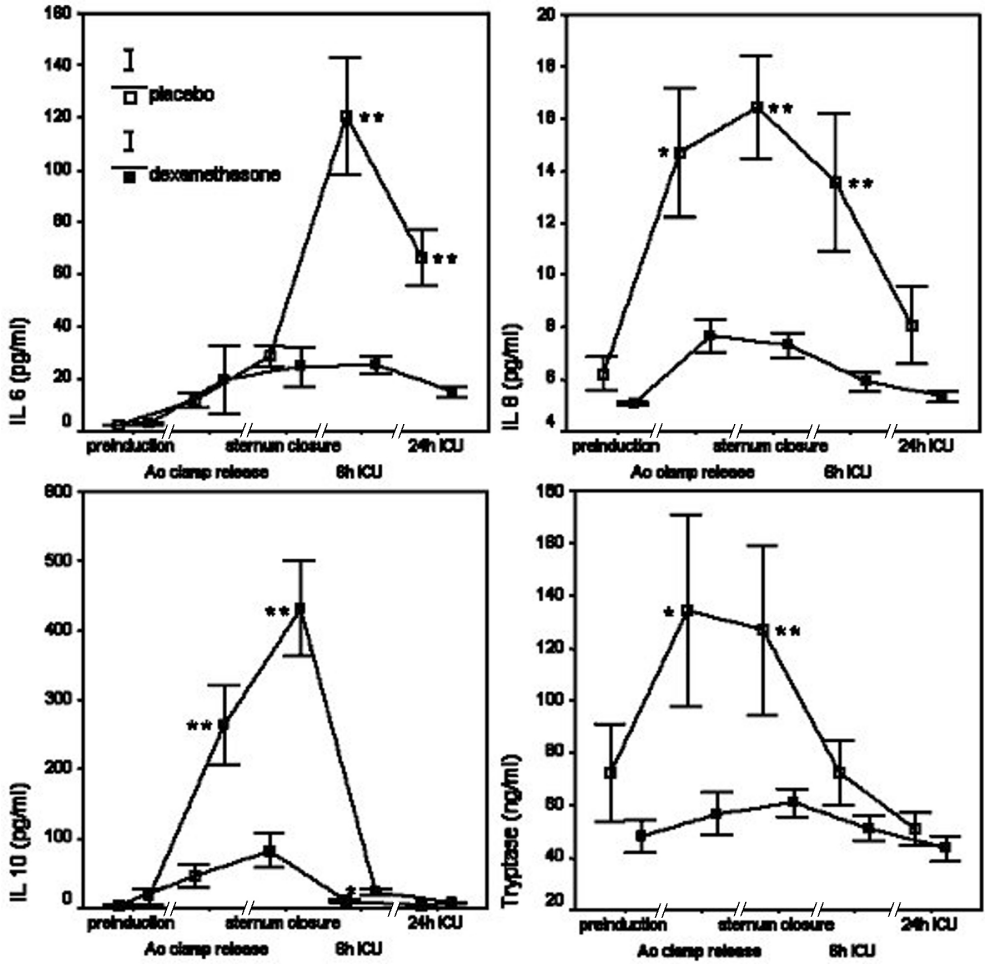

*Figure 2.1: Pro–inflammatory interleukins (Interleukin–6, Interleukin–8), anti–inflammatory interleukin (Interleukin–10), and mast cell degranulation product (Tryptase). The values are represented as mean (symbols) and standard error of the mean (bars). **, * Differences between groups are significant at the .01, .05 level, respectively.*

Renal injury biomarkers

Glomerular and tubular function in this very group of patients was recently described elsewhere[19]. Briefly, urinary NAG increased significantly in time (Wilks p=0.009),

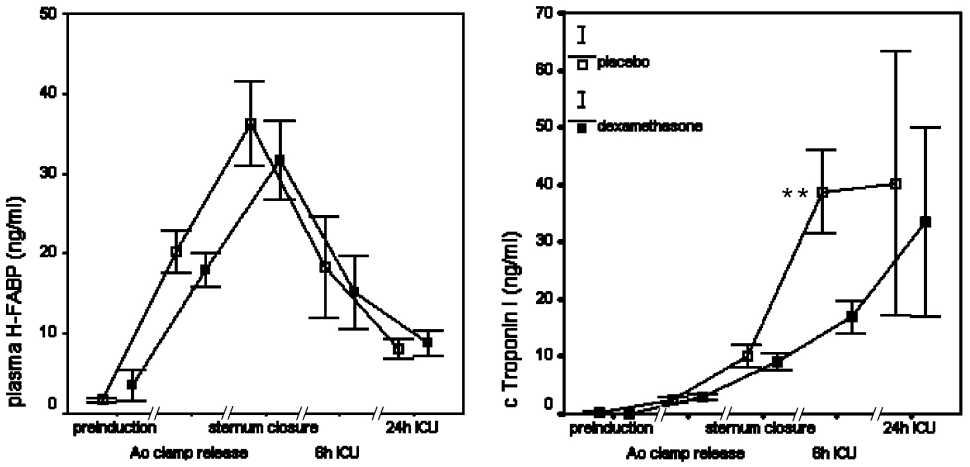

*Figure 2.2: Myocardial injury biomarkers: Heart–type fatty acid binding protein (H–FABP) and cardiac Troponin I (cTnI). The values are represented as mean (symbols) and standard error of the mean (bars). ** Differences between groups are significant at the .01 level.*

reaching peak values at 2 h ICU, with no significant effect for the dexamethasone treatment (Fig. 2.3).
Microalbuminuria increased during CPB, with peak values in the urine collected during CPB for both groups (mean 7.9 mg/mmol creatinine, 95%CI=(4.8–10.9)).

Intestinal injury biomarkers

Urinary I–FABP and L–FABP (Fig. 2.4a,b) increased significantly during CPB (Wilks' p=0.02 I–FABP, and p=0.013 L–FABP) in both groups, reaching peak values in the urine collected during the first postoperative 2 h and 6 h, respectively. The change in mean urinary L–FABP production was significantly dependent upon dexamethasone treatment (Wilks' p=0.026), with higher values in the dexamethasone group. Analyzing each individual time point, no statistical significant differences between groups were detected for I–FABP and L–FABP.

Hepatic injury biomarkers

αGST increased promptly after initiation of CPB in both groups, with peak values during sternum closure (Wilks' $p<0.001$) (Fig. 2.5). There were no differences between the groups (time points and total AUC). ALT remained constant for the duration of the investigation. AST increased moderately in both groups with maximum values

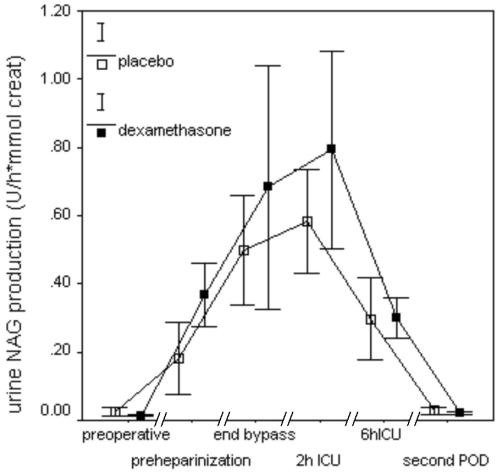

Figure 2.3: Renal injury biomarker: urine N–acetyl–glucosaminidase (NAG). The values are represented as mean (symbols) and standard error of the mean (bars)[19].

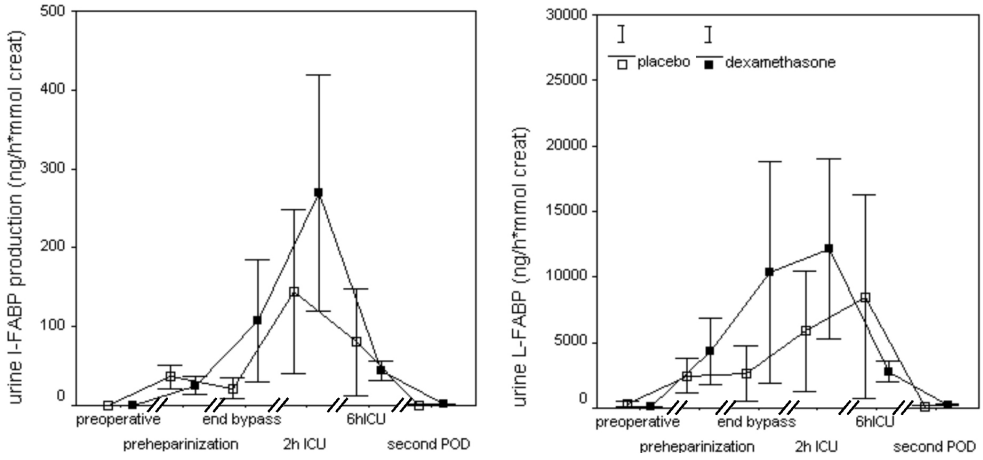

Figure 2.4: Intestinal injury biomarkers: urine intestinal–type (I–FABP) and liver–type (L–FABP) fatty acid binding proteins. The values are represented as mean (symbols) and standard error of the mean (bars).

at 24 h ICU (58.9±10.8 U/L).

Serum glucose (Fig. 2.6). Dexamethasone treatment increased significantly the serum

glucose levels (p=0.009). During sternum closure the values reached the peak values of 10.7±0.6 mmol/L in the dexamethasone group, and 7.4±0.5 mmol/L in the placebo group. The glucose values in the dexamethasone group were significantly higher than in the placebo group during sternum closure (p=0.005), 6 h ICU (p=0.007) and 24 h ICU (p=0.023).

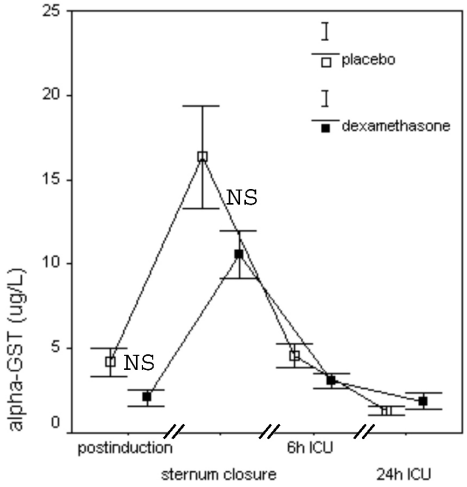

Figure 2.5: Hepatic injury biomarker: plasma αGlutathione S–transferase (αGST). The values are represented as mean (symbols) and standard error of the mean (bars). NS=not significant.

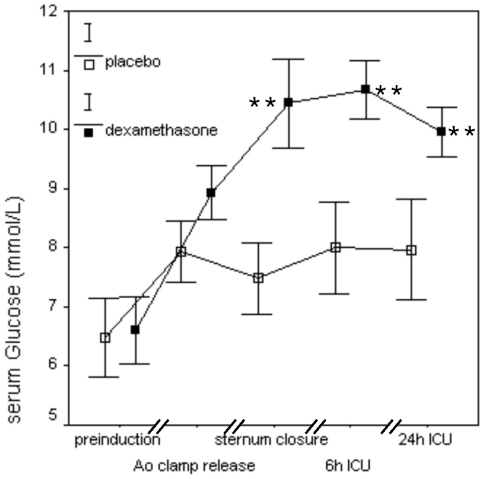

*Figure 2.6: Serum glucose level. The values are represented as mean (symbols) and standard error of the mean (bars). **, * Differences between groups are significant at the .01, .05 level, respectively.*

Predictors of organ injury

Peak serum glucose values (mmol/L) were significant independent predictors for urine I–FABP peak values (R^2=42.5%, regression coefficient B=114.4±31.4, Sig.=0.002), urine L–FABP peak values (R^2=47.3%, regression coefficient B=7714.1±1920.9, Sig.=0.001), urine H–FABP peak values (R^2=48%, regression coefficient B=2829.5±694.5, Sig.=0.001) and urine NAG peak values (R^2=32.1%, regression coefficient B=0.21±0.07, Sig.=0.009).

Perfusion duration (minutes) was a significant independent predictor for urine I–FABP peak values (R^2=22%, regression coefficient B=6.7±3, Sig.=0.03), urine L–FABP peak values (R^2=26.6%, regression coefficient B=476±186.4, Sig.=0.02), and urine H–FABP (R^2=37.7%, regression coefficient B=206.3±62.5, Sig.=0.004).

Correlations

Inflammatory biomarkers: The statistical correlations found between the inflammatory markers are shown in Table 2.2. CRP at 6 h ICU correlated positively with peak cTnI concentrations (corr. 0.49, p=0.02). Tryptase peak values correlated negatively with peak plasma I–FABP (corr. –0.445, p=0.04), peak urinary I–FABP (corr. –0.474, p=0.03), peak urinary L–FABP (corr. –0.647, p=0.002), peak urinary H–FABP (corr. –0.60, p=0.005), peak urinary NAG (corr. –0.609, p=0.004), peak microalbuminuria (corr. –0.559, p=0.01).

Spearman's corr.	**CRP 6h ICU**	**Peak Tryptase**		
Peak IL–6	.568**	.474*	**Peak IL–6**	
Peak IL–8	.498*	.520*	.555*	**Peak IL–8**
Peak IL–10	ns	–.411 (p=0.072)	–.412 (p=0.071)	–.630**

*Table 2.2: Statistical correlations (non-parametric Spearman's correlation) between the peak values of the pro inflammatory (Interleukin–6–IL–6,Interleukin–8–IL–8), anti–inflammatory interleukins (Interleukin–10–IL–10) and tryptase. * Correlation is significant at the .05 level (2–tailed), ** Correlation is significant at the .01 level (2–tailed), ns=not significant.*

Myocardial biomarkers: cTnI AUC correlated significantly with plasma H–FABP (AUC corr. 0.469, p=0.03; peak corr. –0.444, p=0.05) and CK–MB (AUC corr. 0.80, p<0.001, peak corr. 0.77, p<0.001).

Intestinal biomarkers: Urine I–FABP correlated significantly with urine L–FABP (peak corr. 0.81, p<0.001).

Renal biomarkers: the urinary peak of H–FABP correlated strongly and significantly with the urinary peak of NAG (corr. 0.65, p=0.002) and peak microalbuminuria (corr. 0.66, p=0.001). In addition, the peaks of intestinal damage markers correlated significantly with the peak values of renal damage markers (I–FABP∼NAG: corr. 0.55, p=0.01; I–FABP∼H–FABP corr. 0.77, p<0.001; L–FABP∼Microalbuminuria corr. 0.57, p=0.009).

2.4 Discussion

In the present study we have found that administration of dexamethasone inhibited the SIRS in patients undergoing elective on–pump CABG. However, administration of dexamethasone did not offer protection against pulmonary, renal and intestinal perioperative damage. Even more, dexamethasone–induced hyperglycemia was found as a strong independent predictor of intestinal and renal perioperative damage. Postoperative pulmonary function was adversely affected by dexamethasone, with decreased PaO_2/FiO_2 ratio and prolonged time to tracheal extubation in the dexamethasone group of patients.

Myocardial injury
Dexamethasone seemed to offer, to a small extent, myocardial protection during the first 6 h of reperfusion as shown by lower concentration of cardiac Troponin I, but with no further protection after 24 h of reperfusion. Additionally, the protective effect was not noticeable when estimating the myocardial damage by the plasma concentration of heart–type fatty acid binding protein. The recently introduced marker H–FABP is a cytosolic protein abundant in the myocardium, with a 10 fold lower expression in the skeletal muscles, kidney (distal tubules), lung, brain and endothelial cells[20,21]. H–FABP has been introduced as a plasma marker for an early assessment of myocardial tissue injury with a peak as early as 3 h after acute myocardial infarction and 2 h post–reperfusion after CABG[22,23]. The early plasma peak also present in our study promotes H–FABP as a valuable myocardial injury marker, since peak levels of the Troponine T and I occur only much later, around 18 hours post reperfusion[24].

Pulmonary Injury
Dexamethasone treatment resulted in more pronounced postoperative pulmonary dysfunction and prolonged time to tracheal extubation. The detrimental consequence of dexamethasone on lung function was clinically relevant in terms of significantly lower PaO_2/FiO_2 ratio immediately after extubation, and the significantly prolonged time to tracheal extubation in the patients in the dexamethasone group. These adverse effects of dexamethasone treatment on pulmonary function confirm the findings reported recently by Chaney et al.[12,13], after treatment with methylprednisolon in a similar group of patients.

Renal Injury
Urinary N–acetyl–glucosaminidase (enzyme released from injured proximal renal tubules) and microalbuminuria increased significantly during CPB, with no effect of dexamethasone. Measurements of urinary H–FABP proved to be a better indication of kidney damage than of myocardial damage, because the urinary peak of H–FABP did not correlate with the other cardiac markers, but correlated strongly and significantly with the urinary peak of NAG (proximal tubules injury) and peak

microalbuminuria (glomerular injury). This measurement might be explained by a urinary release of H–FABP from the damaged distal renal tubules. H–FABP has been associated before with early release following injury of the distal renal tubules[25,26]

Intestinal injury
I/L–FABP are cytosolic proteins readily released into the circulation following enterocytes damage, with a 40 fold higher content of L–FABP, reported as useful urine markers for the detection of intestinal injury[27–29]. Elevated I–FABP in relation to gastrointestinal complications following cardiopulmonary bypass was described earlier[29]. The increased values of I/L–FABP during CPB reported in our study confirm the indirect line of evidence suggesting mucosal integrity loss during CPB reported previously as a reduction in intramucosal pH, increase in gut permeability and endogenous endotoxemia[30–32]. Significantly elevated I–FABP urine levels in critical ill patients correlated with clinical development of the systemic inflammatory response syndrome[33]. In our study, 20% in the variation of intestinal injury markers and 30% in the variation of renal injury markers were explained by the CPB duration.

Hepatic injury
αGlutathione S–transferase (αGST) increased promptly after initiation of CPB in both groups, with peak values during sternum closure, without effect for the dexamethasone treatment. Increased levels of αGST as indication of hepatocytes injury were reported before in patients undergoing CPB[34].

Inflammatory response
The release of pro–inflammatory interleukins was inhibited by dexamethasone, while the anti–inflammatory interleukin IL–10 was increased. The acute phase protein CRP was found in lower concentrations during the first day postoperative in the plasma of the patients receiving dexamethasone. These data confirmed that the administered dose of dexamethasone (1 mg/kg before induction of anesthesia and 0.5 mg/kg after 8 hours) was therapeutically effective. In the first 24 postoperative hours, rectal temperature was moderately but significantly higher in the placebo group. In a recent study postoperative temperature was controlled by active surface cooling to prevent cerebral damage[35]. The present study demonstrates that temperature can be controlled as effectively with medication. The modulation of the humoral inflammatory response and lower postoperative rectal temperatures as a result of dexamethasone treatment observed in this study are in agreement with previous studies published on the subject. Glucocorticoid administration prior to CPB was shown to attenuate inflammatory response, as based on biochemical analysis of serum inflammatory mediators, to reduce the incidence of postoperative febrile episodes in pediatric cardiac surgery[36] and to decrease incidence of postoperative hyperthermia in adult surgery[11]. Only limited amount of data characterizing mast cell activation with subsequent

tryptase release during CPB is available in the literature[37,38]. The present study reports an important mast cell degranulation (activation), with a peak in the systemic release of tryptase as early as the release of the aorta cross clamp. Dexamethasone was effective in inhibiting tryptase release. Tryptase is a serine proteinase with trypsin–like properties, being released in peripheral blood subsequent to mast cell activation in lungs, heart, stomach, spleen, skin, colon and kidneys[39,40]. Extracellular release of tryptase is known to recruit inflammatory cells, to induce IL–8 secretion from airway epithelial cells, and to promote airway inflammation[41]. In our study, tryptase correlated positively and significantly with IL–6 and IL–8 (Table 2.2). Surprisingly, we also found a negative correlation between tryptase and the organ damage markers. Lower levels of tryptase correlated significantly with higher levels of intestinal injury (plasma I–FABP, urinary I–FABP, urinary L–FABP), and high levels of proximal tubular (urine NAG), distal tubular (urine H–FABP) and glomerular (microalbuminuria) renal damage during the first two hours of reperfusion post CPB.

These data support the hypothesis of a preconditioning effect of tryptase: early release of tryptase might offer protection against perioperative intestinal and renal damage. By amplifying the signal for histamine release[42], and thus inducing an endothelial–NO dependent vasodilator effect, tryptase might counteract the vasoconstriction induced by the hyperglycemia and ischemia/reperfusion injury. This hypothesis is supported by data showing that histamine–induced vasodilatation mediated by endothelial derived NO was attenuated under hyperglycemic conditions[43]. In our study, we found high serum glucose levels in patients undergoing CPB receiving dexamethasone. Serum glucose levels had strong positive predictive value for the postoperative intestinal and renal damage. The variation in serum glucose concentration explained more than 40% in the variation of intestinal damage biomarkers (I–FABP and L–FABP) and more than 30% in the variation of renal tubules damage markers (NAG and urine H–FABP). In addition, the patients in the dexamethasone treated group tended to require more insulin treatment.

To explain our results on the effect of acute hyperglycemia on organ injury, we refer to the recent published results of Vanhorebeek et al.[44], showing in a study on critically ill patients that hyperglycemia was associated with organ injury, as demonstrated by mitochondrial ultrastructural abnormalities with increased production of reactive oxygen species in the hepatocyte of hyperglycemic patients (10–11.1 $\mathrm{mmol.L^{-1}}$). Using animal experiments, Bohlen and colleagues[45,46] demonstrated that oxygen radicals formed during acute hyperglycemia affect flow–mediated endothelium regulation in the intestinal vasculature due to depression of nitric oxide, resulting in reduced blood flow.

Our data quantifies for the first time the effect of hyperglycemia on organ injury. These results might provide an explanation for the increased morbidity and mortality among critically ill patients in the surgical intensive care unit when blood glucose

level is above 6.1 $.L^{-1}$ [47]. A limitation of this study is that, despite randomization, patient characteristics were slightly different. In the dexamethasone group patients were slightly older and therefore the possibility of confounding exists. However, this influence seems limited because age did not prove to be a predictor for any of the biomarkers tested. Moreover, baseline values of a large number of sensitive markers were similar in both groups, and there was no correlation between age and the baseline levels of the tested markers. The patients in this study had little co–morbidity and thus belong to the "healthy" CABG group. Dexamethasone in patients of a higher risk profile could have different effects on inflammatory response and organ injury. Finally, this study was not powered to analyze effects on mortality, or possible differences in wound healing and postoperative infections.

2.5 Conclusions

Dexamethasone, as administered in this study, offered no protection against transient, perioperative renal, intestinal and hepatic injury in patients undergoing on–pump CABG. Dexamethasone treatment resulted in more pronounced postoperative pulmonary dysfunction, prolonged time to tracheal extubation and initiated postoperative hyperglycaemia. Given the strong positive predictive value of hyperglycaemia for renal and intestinal tissue injury, a stricter management of serum glucose may offer beneficial effects.
As a contribution to the efforts made for understanding the complex pathophysiologic mechanism of the "post–CPB" syndrome, this study verified theories existent in the literature and brought under attention new essential aspects: (1) higher glycemic values as strong predictors for higher intestinal and renal damage; (2) preconditioning effect of mast cells activation and tryptase release for the subsequent postoperative intestinal and renal injury.

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References

1. Chaney MA. Corticosteroids and cardiopulmonary bypass: a review of clinical investigations. *Chest* 2002;121:921–31.

2. Paparella D, Yau TM, Young E. Cardiopulmonary bypass induced inflammation: pathophysiology and treatment. An update. *Eur. J. Cardiothorac Surg.* 2002;21:232–44.

3. Kloner RA, Jennings RB. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications – part 1. *Circulation.* 2001;104:2981–2989.

4. Ng CS, Wan S, Yim AP, et al. Pulmonary dysfunction after cardiac surgery. *Chest* 2002;121:1269–77.

5. Halm MA. Acute gastrointestinal complications after cardiac surgery. *Am. J. Crit. Care.* 1996;5:109–18.

6. Nakamura K, Harasaki H, Fukumura F, et al. Comparison of pulsatile and non–pulsatile cardiopulmonary bypass on regional renal blood flow in sheep. *Scand. Cardiovasc. J.* 2004;38:59–63.

7. Jansen NJ, van Oeveren W, van den Broek L, et al. Inhibition by dexamethasone of the reperfusion phenomena in cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg.* 1991;102:515–25.

8. El Azab SR, Rosseel PM, de Lange JJ, et al. Dexamethasone decreases the pro– to anti–inflammatory cytokine ratio during cardiac surgery. *Br. J. Anaesth.* 2002;88:496–501.

9. von Spiegel T, Giannaris S, Wietasch GJ, et al. Effects of dexamethasone on intravascular and extravascular fluid balance in patients undergoing coronary bypass surgery with cardiopulmonary bypass. *Anesthesiology* 2002;96:827–34.

10. Kawamura T, Inada K, Okada H, et al. Methylprednisolone inhibits increase of interleukin 8 and 6 during open heart surgery. *Can. J. Anaesth.* 1995;42:399–403.

11. Toft P, Christiansen K, Tonnesen E, et al. Effect of methylprednisolone on the oxidative burst activity, adhesion molecules and clinical outcome following open heart surgery. *Scand. Cardiovasc. J.* 1997;31:283–8.

12. Chaney MA, Durazo–Arvizu RA, Nikolov MP, et al. Methylprednisolone does not benefit patients undergoing coronary artery bypass grafting and early tracheal extubation. *J. Thorac. Cardiovasc. Surg.* 2001;121:561–569.

13. Chaney MA, Nikolov MP, Blakeman BP, et al. Hemodynamic effects of methylprednisolone in patients undergoing cardiac operation and early extubation. *Ann. Thorac. Surg.* 1999;67:1006–1011.

14. Payne V, Kam PC. Mast cell tryptase: a review of its physiology and clinical significance. *Anaesthesia.* 2004;59:695–703.

15. van der Maaten JM, Epema AH, Huet RC, et al. The effect of midazolam at two plasma concentrations of hemodynamics and sufentanil requirement in coronary artery surgery. *J. Cardiothorac. Vasc. Anesth.* 1996;10:356–63.

16. Lockwood TD, Bosmann HB. The use of urinary N–acetyl–β–glucosaminidase in human renal toxicology: II. Partial biochemical characterization and excretion in humans and release from the isolated perfused rat kidney. *Toxicol. Appl. Pharmacol.* 1979;49:323–36.

17. Gollin G, Marks C, Marks W. Intestinal fatty acid binding protein in serum and urine reflects early ischemic injury to the small bowel. *Surgery.* 1993;113:545–51.

18. Beckett GJ, Hayes JD. Glutathione S–transferases: biomedical applications. *Adv. Clin. Chem.* 1993;30:281–380.

19. Loef BG, Henning RH, Epema AH, et al. Effect of dexamethasone on perioperative renal function impairment during cardiac surgery with cardiopulmonary bypass. *Br. J. Anaesth.* 2004;93:793–8.

20. Veerkamp JH, Paulussen RJ, Peeters RA, et al. Detection, tissue distribution and (sub)cellular localization of fatty acid–binding protein types. *Mol. Cell Biochem.* 1990;98:11–8.

21. Yoshimoto K, Tanaka T, Somiya K, et al. Human heart–type cytoplasmic fatty acid–binding protein as an indicator of acute myocardial infarction. *Heart Vessels.* 1995;10:304–9.

22. Petzold T, Feindt P, Sunderdiek U, et al. Heart–type fatty acid binding protein (H–FABP) in the diagnosis of myocardial damage in coronary artery bypass grafting. *Eur. J. Cardiothorac. Surg.* 2001;19:859–64.

23. Suzuki K, Sawa Y, Kadoba K, et al. Early detection of cardiac damage with heart fatty acid–binding protein after cardiac operations. *Ann. Thorac. Surg.* 1998;65:54–8.

24. Swaanenburg JC, Loef BG, Volmer M, et al. Creatine kinase MB, troponin I, and troponin T release patterns after coronary artery bypass grafting with or without cardiopulmonary bypass and after aortic and mitral valve surgery. *Clin. Chem.* 2001;47:584–7.

25. K. Lam, S. Borkan, K. Claffey, et al. Properties and differential regulation of two fatty acid binding proteins in the rat kidney. *J. Biol. Chem.* 1988;263:15762-15768.

26. Gok MA, Pelzers M, Glatz JF, et al. Do tissue damage biomarkers used to assess machine–perfused NHBD kidneys predict long-term renal function post-transplant? *Clin. Chim. Acta* 2003;338:33–43.

27. elsers MM, Namiot Z, Kisielewski W, et al. Intestinal–type and liver–type fatty acid–binding protein in the intestine. Tissue distribution and clinical utility. *Clin. Biochem.* 2003;36:529–35.

28. Lieberman JM, Sacchettini J, Marks C, et al. Human intestinal fatty acid binding protein: report of an assay with studies in normal volunteers and intestinal ischemia. *Surgery* 1997;121:335–42.

29. Holmes JH 4th, Lieberman JM, Probert CB, et al. Elevated intestinal fatty acid binding protein and gastrointestinal complications following cardiopulmonary bypass: A preliminary analysis. *J. Surg. Res.* 2001;100:192–6.

30. Sinclair DG, Haslam PL, Quinlan GJ, et al. The effect of cardiopulmonary bypass on intestinal and pulmonary endothelial permeability. *Chest* 1995;108:718–24.

31. Ohri SK, Bjarnason I, Pathi V, et al. Cardiopulmonary bypass impairs small intestinal transport and increase gut permeability. *Ann. Throrac. Surg.* 1993;55:1080–6.

32. Wan S, LeClerc JL, Huynh CH, et al. Does steroid pretreatment increase endotoxin release during clinical cardiopulmonary bypass? *J. Thorac. Cardiovasc. Surg.* 1999;117:1004–8.

33. Lieberman JM, Marks WH, Cohn S, et al. Organ failure, infection, and the systemic inflammatory response syndrome are associated with elevated levels of urinary intestinal fatty acid binding protein: study of 100 consecutive patients in a surgical intensive care unit. *J. Trauma.* 1998;45:900–6.

34. Kumle B, Boldt J, Suttner SW, et al. Influence of prolonged cardiopulmonary bypass times on splanchnic perfusion and markers of splanchnic organ function. *Ann. Thorac. Surg.* 2003;75:1558–64.

35. Bar-Yosef S, Mathew JP, Newman MF, et al. Prevention of Cerebral Hyperthermia During Cardiac Surgery by Limiting On–Bypass Rewarming in Combination with Post–Bypass Body Surface Warming: A Feasibility Study. *Anesth. Analg.* 2004;99:641–646.

36. Bronicki RA, Backer CL, Baden HP, et al. Dexamethasone reduces the inflammatory response to cardiopulmonary bypass in children. *Ann. Thorac. Surg.* 2000;69:1490–5.

37. Withington DE, Aranda JV. Histamine release during cardiopulmonary bypass in neonates and infants. *Can. J. Anaesth.* 1997;44:610–6.

38. an Overveld FJ, De Jongh RF, Jorens PG, et al. Pretreatment with methylprednisolone in coronary artery bypass grafting influences the levels of histamine and tryptase in serum but not in bronchoalveolar lavage fluid. *Clin. Sci. (Lond).* 1994;86:49–53.

39. Wang HW, McNeil HP, Husain A, et al. Delta tryptase is expressed in multiple human tissues, and a recombinant form has proteolytic activity. *J. Immunol.* 2002;169:5145–52.

40. Ehara T, Shigematsu H. Mast cells in the kidney. *Nephrology (Carlton).* 2003;8:130–138.

41. Huang C, Friend DS, Qiu WT, et al. Induction of a selective and persistent extravasation of neutrophils into the peritoneal cavity by the tryptase mouse mast cell protease 6. *J. Immunol.* 1998;160:1910.

42. He S, Walls AF. Human mast cell tryptase: a stimulus of microvascular leakage and mast cell activation. *Eur. J. Pharmacol.* 1997;328:89–97.

43. Yousif MH, Oriowo MA, Cherian A, et al. Histamine–induced vasodilatation in the perfused mesenteric arterial bed of diabetic rats. *Vascul. Pharmacol.* 2002;39:287–92.

44. Vanhorebeek I, De Vos R, Mesotten D, et al. Protection of hepatocyte mitochondrial ultrastructure and function by strict blood glucose control with insulin in critically ill patients. *Lancet.* 2005;365:53–9.

45. Bohlen HG, Lash JM. Topical hyperglycemia rapidly suppresses EDRF–mediated vasodilation of normal rat arterioles. *Am. J. Physiol.* 1993;265:H219–25.

46. Jin JS, Bohlen HG. Non–insulin–dependent diabetes and hyperglycemia impair rat intestinal flow–mediated regulation. *Am. J. Physiol.* 1997;272:H728–34.

47. van den Berghe G, Wouters P, Weekers F, et al. Intensive insulin therapy in the critically ill patients. *N. Engl. J. Med.* 2001;345:1359–67.

Chapter 3

Organ Perfusion During Cardiopulmonary Bypass: Hematocrit, Blood Transfusion and Temperature Effect on Organ Viability.

Combined Strategy to Limit Perioperative Myocardial, Renal and Intestinal Tissue Injury in Patients Undergoing On–pump Coronary Artery Bypass Grafting.

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Abstract

Study Objectives

An experimental operative protocol was developed aiming to eliminate several potential stress factors that might lead to injury and dysfunction during coronary artery bypass grafting (CABG) with cardiopulmonary bypass (CPB): (1) cold–crystalloid cardioplegia and atrial intracavitary cooling, (2) autologous priming and partial recovery of the cardioplegic fluid; (3) corporeal normothermia.

Methods

Prospective, pseudo–double blind, randomized clinical trial, investigating the clinical benefits of a new experimental intraoperative protocol. Clinical outcome and transient postoperative injury of the myocardium (creatine kinase–MB, CK–MB), renal tubules (urine N–acetyl–glucosaminidase, NAG) and small–intestine (intestinal–fatty–acid–binding–protein, I–FABP) were investigated.

Results

Hematocrit values during, and immediately postoperative (experimental group 27.8±0.6%; standard group 24.4±0.7%) were significantly different (p=0.002). Postoperative CK–MB, NAG and I–FABP were significantly lower in the experimental group. In the standard hypothermic group of patients, the rectal temperatures measured after 60 min of CPB correlated negatively with postoperative I–FABP. The hematocrit explained 38% and 37% in the variability of postoperative NAG and I–FABP, respectively. An extra 21.5% of the NAG variability and 10% of the I–FABP variability, that could not be explained by the variation in hematocrit, were explained by the blood–transfusion requirements

Conclusions

The addition of an intracavitary cooling system to the standard cold crystalloid cardioplegia during on–pump CABG offered a better protection of the myocardium, with decreased postoperative plasma CK–MB. Partial recovery of intra–atrial cardioplegic fluid and autologous–blood priming limited the extent of intra–operative hemodilution and blood transfusion requirements. An important attenuation of the transient renal and intestinal postoperative injury was observed. Additional protection on renal and intestinal injury was achieved by corporeal normothermia.

3.1 Introduction

The available scientific data concerning the effectiveness and safety of cardiopulmonary bypass (CPB) for patients undergoing coronary artery bypass grafting (CABG) brings to attention several key principles to serve as basis for practical guidelines. Encouraged and generally accepted techniques are cold crystalloid cardioplegia, mild corporeal hypothermia and hemodilution to a hematocrit of 23%.

Myocardial protection against ischemic injury during heart arrest and aorta cross clamping is achieved by hypothermia, which reduces oxygen demands and prolongs tolerable ischemic arrest time. Hypothermia can be induced using various techniques, such as cold saline/ice flush in the pericardial sac, cold crystalloid cardioplegia (4° C, high potassium, cold fluid perfused through coronary arteries), cooling jackets (cold fluid perfused through a very small cooling mattress that is pressed directly against the outside of the heart) and intracavitary cooling (intern cooling of the heart chambers).

Systemic protection against global ischemic injury during extracorporeal circulation: contrary to conventional thinking about the benefits of corporeal hypothermia, an increasing number of clinical studies support corporeal normothermia. It was demonstrated that hypothermic CPB is responsible for a greater platelet activation and endothelial dysfunction than normothermic CPB, leading to more profound changes in the hemostatic and inflammatory systems[1]. Furthermore, a significant positive influence of normothermic CPB temperature was registered on perfusion management, postoperative hemodynamics and blood loss[2].

Normothermic cerebral protection during CPB was also confirmed by studies showing similar patterns of S–100β release[3] and less pronounced subclinical impairment of cognitive brain function in patients undergoing normothermic CPB as compared with mildly hypothermic CPB[4,5].

Hemodilution is encouraged by the current CPB management guidelines, with the rationale based on the reduction of blood viscosity by hemodilution, and thus an improved regional blood flow in the setting of hypoperfusion and hypothermia[6]. However, excessive hemodilution may lead to organ ischemia via a reduction of oxygen–carrying capacity uncompensated by autoregulatory and/or rheologic increase in organ blood flow. In animal models, magnetic resonance and near–infrared spectroscopy suggested that brain injury might be caused by hypoxic–ischemic injury as a result of currently recommended protocols for hemodilution during CPB[7]. In clinical studies, a significant independent association was found between the lowest hematocrit during bypass and acute renal injury[8], with significant benefits on renal function after reduction of the bypass prime volume[9].

Besides lowering the oxygen carrying capacity, hemodilution was shown to be responsible of endothelial cell activation during CPB. By decreasing red blood cell aggregation and plasma viscosity, hemodilution is expected to modify homeo–rheological variables responsible for a constant shear stress at the endothelial wall, inducing mechanical endothelial activation[10].

Our therapeutic strategy aimed to explore, advance and optimize simultaneously more than one method of protection by eliminating several potential stress factors that might lead to injury and dysfunction during CPB: inadequate hypothermic cardioplegia, excessive hemodilution with subsequent need for perioperative blood transfusion, and corporeal hypothermia.
An experimental operative protocol was developed to meet multiple objectives: (1) homogeneous cooling of the myocardium by combining cold crystalloid cardioplegia technique with intracavitary cooling of the heart; (2) prevention of excessive hemodilution by autologous priming of the extracorporeal circuit and partial recovery of the cardioplegic fluid; (3) corporeal normothermia, possible on the account of a more efficient topical cooling of the heart. The consequences on the postoperative organ injury and clinical outcome of the patients were investigated.

3.2 Patients, Materials and Methods

The clinical study was performed at the VU University Medical Center Amsterdam, The Netherlands.
The study was designed as a prospective, pseudo–double blind (blinding of the patient and lab investigator), randomized clinical trial, investigating the clinical benefits of a new experimental intraoperative protocol that combined cold crystalloid cardioplegia with intracavitary cooling, autologous priming and corporeal normothermia.
After approval by the hospital ethics committee, patients scheduled for elective first time CABG were prospectively screened according to entry criteria. All patients included (n=40) in the study were 45 to 70 years of age, had coronary artery disease with normal renal function (as assessed by a serum creatinine lower than 150 μmol.liter^{-1} and normal urinalysis), normal hepatic, cerebral and cardiac function (ejection fraction $> 45\%$). Written informed consent forms were obtained in all cases.
Patients with preoperative immunosuppressive therapy, preoperative use of NSAID, intra aortic balloon support, requiring aneurysmectomy, insulin–dependent diabetes mellitus, recent myocardial infarction, unstable angina, or recent use of radiocontrast were excluded.

Anaesthetic management

On the day of operation, patients received their usual early morning dose of antianginal medication, 5 mg of lorazepam, but no diuretics were administered. Anesthesia was induced using 3–7 μg.kg^{-1} intravenous sufentanil forte, 0.1 mg.kg^{-1} pancuroniumbromide and 0.1 mg.kg^{-1} midazolam. General anesthesia was maintained by a continuous infusion of propofol 5–15 mL.h^{-1} (20 mg.mL^{-1}). After endotracheal intubation, patients were ventilated using an inspiratory mixture of 50% oxygen and 50% of air at a frequency of 14–18 breaths.min^{-1} and 5 cm H_2O of PEEP.
After induction of anesthesia patients received 1 mg.kg^{-1} dexamethasone and 1500 mg

cefuroxime. Radial artery and thermodilution pulmonary artery catheters were inserted for hemodynamic monitoring and blood sampling.
A continuous positive airway pressure of 5 cm H_2O and a FiO_2 of 21% was maintained in the lungs during CPB. Nitroglycerin infusion of 1 mg.kg^{-1}.min^{-1} was started in the rewarming period. In case of hemodynamic instability, fluid replacement and inotropic support with dopamine (4 mg.ml^{-1}) and/or nitroglycerin were the first steps taken to stabilize the patient. The mean arterial blood pressure was maintained between 65 and 80 mmHg, pulmonary artery wedge pressure between 8 and 12 mmHg.
Transfusion of packed cells were given at a hemoglobin < 4.5 mmol.L^{-1}.

Extracorporeal circuit

In both groups we used a S3 heart–lung machine (Stöckert Instrumente GmbH, Munich, Germany) with a centrifugal pump (Delphin, Terumo Europe NV, Leuven, Belgium), a heat exchange device (Stöckert Instruments GmbH), polyvinyl tubing system (Medtronic Inc., Minneapolis, MN, USA), a hollow fiber oxygenator (Affinity, Medtronic), a soft shell collapsible venous reservoir (MVR 1600, Medtronic), an arterial line filter (Affinity, 38 mic, Medtronic), and a cardiotomy reservoir (Intercept cardiotomy, Medtronic).
In the control group, the total priming volume consisted of 1400 ml: 1000 ml modified fluid gelatin (Gelofusin, Braun, Melsungen, Germany), 50 ml lactated Ringer's solution, 200 ml of aprotinin, 100 ml mannitol and 50 ml sodium bicarbonate (8.4%) containing 1500m g cefuroxime and 5000 IU bovine heparin.
In the experimental group, retrograde autologous priming was performed using 500 ml autologous blood, collected in a transfer bag during the bicaval cannulation procedure. The priming was completed using the priming solution described for the control group. Coronary sinus drainage was achieved by cold crystalloid antegrade cardioplegia (St. Thomas solution) delivery through the aortic root. Use of the experimental cannula allowed partial recovery of the cardioplegic solution. In the middle of the cannula's balloon, on the cardiac site, a side hole is positioned. This specific side hole is connected to a suction line. During and after cardioplegia infusion, the cardioplegia solution (and/or residual systemic blood) from the right atrium was recovered via the suction tubbing.

Cardiopulmonary bypass

Standard group: standard cannulation technique using a two stage venous cannula placed in the inferior vena cava and right atrium.
Experimental group: bicaval cannulation through the superior caval vein using a dual–stage bicaval atrial cooling cannula. This cannula is a cardiopulmonary dual stage venous catheter with an integrated cooling balloon. The system is designed to circulate cold saline (0.9% NaCl) in the right atrium enabling homogeneous cooling of

the atrium. The cannula is a polyvinyl chloride (PVC) fashioned into an "L" shape. An opening at the elbow of the L curve collects venous blood from the superior vena cava. The distal end of the cannula has a cone shape with openings to collect venous blood from the inferior vena cava. Between these two collection areas, a 9 cm long medical grade silicon balloon has been attached. The balloon has a maximum volume capacity of 40 ml. Inflation and deflation of the balloon depends on the saline volume added in the attached closed circuit. Immediately prior to aortic cross–clamping the balloon was filled with saline solution, and thereafter total CPB was initiated. A heat–exchanger (Bentley HE–30) cooled the saline to 4° C and a small roller pump maintained the saline circulation at a flow rate of 300 ml.min^{-1}.
CPB was initiated after systemic heparinization (300 I.U. kg^{-1}), with a celite activated clotting time higher than 480 seconds. The target hematocrit during CPB was higher than 23%. The non–pulsatile blood flow rate was maintained between 2.2–3.0 L.min^{-1}.m^{-2} with mild hypothermia in the standard group (30–33° C), and normothermia (35–36° C) in the experimental group.
Recovered cardioplegia fluid and suctioned blood were discarded.
Electrocardiograms were obtained preoperatively, on intensive care unit admission, and on postoperative days 1 and 2.

Organ injury biomarkers

Myocardial injury biomarker: creatine kinase MB (CK–MB) activity – Vitros analyzer (Ortho Clinical Diagnostics; Beerse, Belgium).
Kidney injury biomarker: urine N–acetyl–glucosaminidase (NAG) release in urine signifies renal damage and ischemia localized at the proximal tubules level[11]. The method of detection was a modified enzyme assay according to Lockwood[12] at pH 4.5 and corrected for non–specific conversion (HaemoScan, Groningen, The Netherlands).
Intestinal injury biomarkers: intestinal fatty acid binding protein I–FABP, ELISA kit (HyCult Biotechnology BV, Uden, The Netherlands) is a cytosolic protein readily released into the circulation following enterocytes damage, reported as sensitive and specific urine markers for the detection of intestinal injury and prediction of gastrointestinal complications after cardiopulmonary bypass[13,14].
Urine NAG and I–FABP concentrations were measured preoperative (baseline) and postoperative, in the urine collected during the first 2 hours postoperative, as previous investigations showed peak values of both markers coming during the first 2 hours after the end of the surgical procedure[15].
Urinary excretions of NAG and I–FABP were calculated as ratio to urine urea concentration in order to correct for urine dilution.
In addition to these organ injury biomarkers, standard laboratory investigation were performed every 24 hours until hospital discharge. Standard laboratory investigations included: hemoglobin, serum glucose, HCO_3^-, sodium ions, potassium ions, urea, serum creatinine, lactate, ASAT, ALAT, C Reactive Protein, total proteins, albumin,

total creatine kinase.
Lactate concentrations in the venous whole blood (Rapidlab 865; Chiron Diagnostics Corp., East Walpole, MA) – end product of anaerobe glycolysis in skeletal muscle, brain and erythrocytes; lactate was measured to give an indication on systemic exposure to hypoxia.

Statistical Analysis

A power analysis based on previous studies in this population on peak postoperative CK–MB plasma activity suggested that at least 40 patients have to be studied in order to detect a 1 SD difference between the two groups, with a reliability of 5% and a power of 80%.
Before analysis, the data was tested for distribution according to Kolmogorov–Smirnov goodness of fit test. Continuous variables were compared by means of parametric (Student T Test) or nonparametric tests (Mann–Whitney). Fisher's exact test was used to compare discrete variables. Correlation between variables was tested using Spearman correlation test. Linear regression analysis was used to detect predictors in the model. Results are presented as mean±SEM (unless stated otherwise). Independent predictors were tested using linear regression analysis. Multivariate analysis was used to find significant predictor models.

3.3 Results

Twenty patients were randomized to each group. Patients' demographic and clinical characteristics, operating times, perioperative fluid management, hemodynamics, coagulation variables and blood loss are presented in Table 3.1. Phenylephrine was administered intraoperatively to three patients in the experimental group (0.83±0.16) and ten patients in the standard group (0.85±0.31), (Fisher's exact test 1–sided p=0.02).
The following complications were diagnosed: re–thoracotomie (n=2, 1 of surgical and 1 of non–surgical etiology) and postoperative atrial fibrillations (n=8). The distribution of complications was similar in both groups. No clinical diagnose of postoperative myocardial infarction, cerebral vascular accidents/transitory cerebral ischemic accidents, acute renal injury or gastrointestinal complications was established. In–hospital mortality was 0% for both groups.

Temperature

The minimum corporeal (intrarectal) temperatures measured during CPB were 32.9±0.2° C in the standard hypothermic group and 35.9±0.1° C in the experimental normothermic group.

In the standard hypothermic group of patients, the rectal temperatures measured after 60 min of CPB correlated negatively with postoperative I–FABP urine concentrations (Spearman's corr. –0.501, p=0.029). In other words, lower rectal temperatures during CPB were associated with higher intestinal damage.

	Standard group Mean±SEM	Experimental group Mean±SEM	P value
No. of patients	20	20	/
Male/Female	18/2	5/5	0.41
Age (y)	60.9±1.8	59.4±1.7	0.55
Body surface area (m^2)	2±0.03	2.03±0.03	0.61
Distal anastomoses per patient	3.7±0.2	4.2±0.1	0.09
Priming volume (ml)	1377±38	857±44	<0.001
Cardioplegic volume (ml)	1382±84	1373±49	0.69
Recovered cardioplegic volume (ml)	0	576±46	/
Fluid balance during CPB	1742±117	771±48	<0.001
Fluid balance 1st POD	1286±240	1091±296	0.41
CPB time (min)	100±5	97±5	0.62
Aorta cross-clamping time (min)	67±5	69±4	0.83
lowest MAP during CPB (mmHg)	53.8±1.4	53.1±1	0.91
CI preoperative (L/min/m^2)	2.43±1.14	2.22±0.13	0.31
CI postoperative (L/min/m^2)	3.2±0.15	3.15±0.19	0.91
CRP preoperative (mg/l)	8.6±4	6.1±1.1	0.41
CRP 2nd POD (mg/l)	60.6±13.3	58.2±8.4	0.78
CRP 3rd POD (mg/l)	94.4±14.2	80.6±13.3	0.44
max ICU APTT (sec)	46.7±1.7	43.8±1.3	0.1
max ICU INR 1h	1.55±0.03	1.58±0.03	0.64
Total blood loss postoperative (ml)	828±89	822±76	0.85
Time to tracheal extubation (h)	10.8±1.11	10.2±0.9	0.71
ICU stay (days)	1.1±0.1	1.05±0.05	0.65

Table 3.1: Patient characteristics and operative data (mean±standard error of the mean). CPB=cardiopulmonary bypass, ICU=intensive care unit, MAP=Mean arterial pressure, CI=cardiac index, APTT=activated partial thromboplastin time, INR=international normalized ration. Statistics: Continuous variables where compared by means of parametric (Student T Test) or nonparametric tests (Mann–Whitney). Fisher's exact test was used to compare discrete variables.

Hematocrit

Hematocrit values were measured systematically for 24 h, starting with a preoperative baseline time point (Fig. 3.1). The values measured in the beginning of CPB ("1st

value during CPB": standard group 24.5±0.7% versus experimental group 28.3±0.7%) and at the end of CPB ("post-CPB": standard group 24.4±0.7% versus experimental group 27.8±0.6%) were significantly different (Mann–Whitney p=0.002 both time points).

Transfusion requirements

Seven patients in the standard group (1.6±0.2 units/patient) and two patients in the experimental group (1 unit/patient) received packed cell (PC) transfusion in the operating room (Fisher's exact test 1–sided p=0.064). In the ICU, twelve patients in the standard group (1.6±0.3 units/patient) and six patients in the experimental group (1.5±0.5 units/patient) received PC transfusion (Fisher's exact test 1–sided p=0.055). No other blood products (single donor plasma or platelets concentrates) were administered in the operation room or ICU to the patients included in this study.

Organ injury biomarkers (Fig. 3.2)

Myocardial injury

Electrocardiographic modifications: ECGs were obtained preoperatively, on intensive care unit admission, and on postoperative days 1 and 2. No postoperative development of new Q waves was registered at any time point.
CK–MB (Fig. 3.2a) increased abruptly during the operation in both groups, with significantly different postoperative CK–MB activity between groups (4 h ICU: Mann–Whitney p=0.021, 8 h ICU: Mann–Whitney p=0.024). The peak values of CK–MB measured after the patients were transferred in the ICU were significantly higher in the standard protocol group than in the experimental protocol group (22.6±2.3 U/l versus, 15.5±1.3 U/l respectively; Mann–Whitney p=0.026).

Renal injury

Serum creatinine values stayed within normal ranges for the entire investigated period, with no differences between groups. The peak values of serum creatinine measured during the investigated period were 91.3±2.1 μmol/l in the standard group, and 93.5±2.3 μmol/l in the experimental group.
Postoperative urine N–acetyl–glucosaminidase (NAG, Fig. 3.2b) concentrations reached significantly higher values in the standard protocol group than in the experimental protocol group (213.6±54.5 mU/mmol urea versus 51.2±11.8 mU/mmol urea, Mann–Whitney p<0.001).

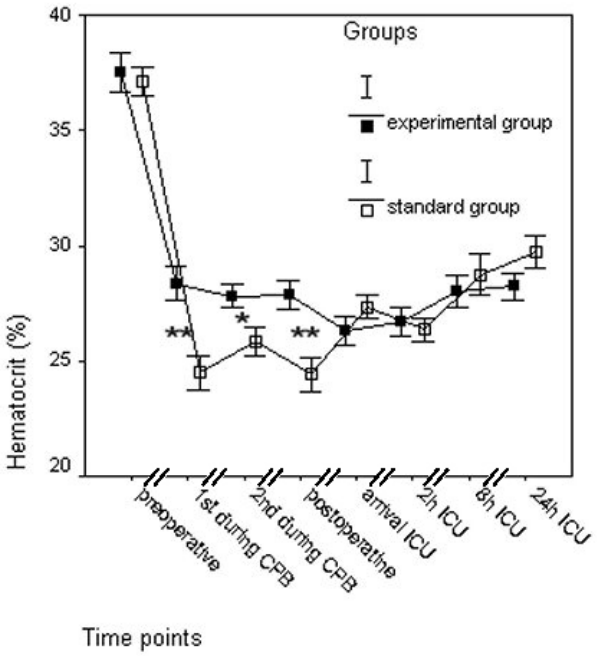

Figure 3.1: Hematocrit measurements in the blood of 40 patients undergoing on–pump CABG according to an experimental CPB protocol (n=20) or the standard CPB protocol (n=20). The values are represented as mean (symbols) and standard error of the mean (bars). * *$p<0.05$,** $p<0.01$*

Intestinal injury

Postoperative urine concentrations of intestinal–type fatty acid binding protein (I–FABP, Fig. 3.2c) was significantly higher in the standard protocol operated patients as compared with values of the patients in the experimental protocol group (325.5±34 versus 181.4±24, Mann–Whitney p=0.003).
Lactate concentrations in the venous whole blood stayed at all time points within normal ranges with no differences between the groups. The lactate values measured during CPB (experimental group 1.07±0.04 mmol/l, standard group 1.16±0.1 mmol/l) correlated positively with the postoperative I–FABP values (Spearman's corr. 0.566, p=0.003) and postoperative NAG values (Spearman's corr. 0.391, p=0.05).

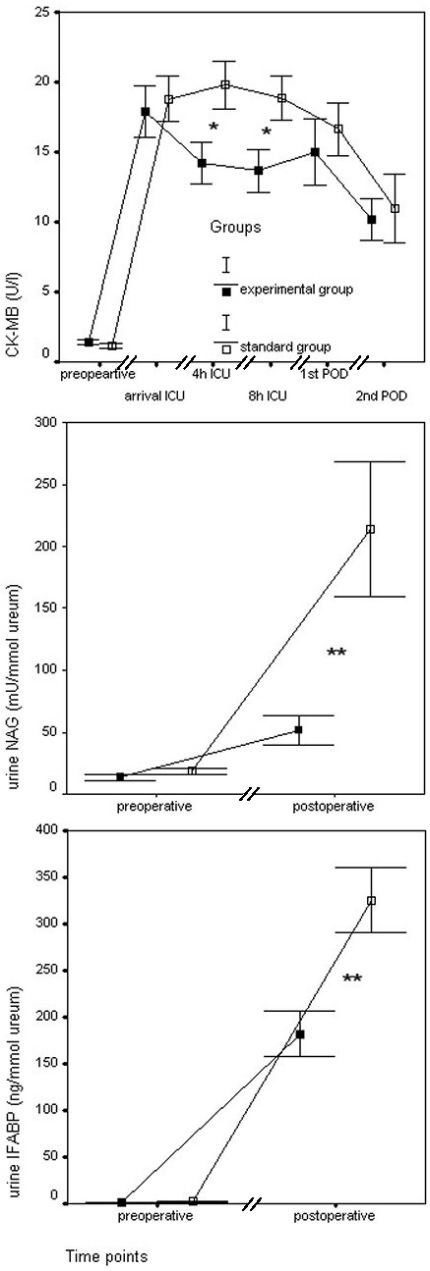

Figure 3.2: **a)** *Creatine kinase–MB (CK–MB) activity in the plasma of 40 patients undergoing on–pump CABG according to an experimental CPB protocol (n=20) or the standard CPB protocol (n=20). The values are represented as mean (symbols) and standard error of the mean (bars).* * $p<0.05$. **b)** *N–acetyl–glucosaminidase (NAG) excretion in the urine of 40 patients undergoing on–pump CABG according to an experimental CPB protocol (n=20) or the standard CPB protocol (n=20). Urinary excretions of NAG was calculated as ratio to urine urea concentration in order to correct for urine dilution. The values are represented as mean (symbols) and standard error of the mean (bars).* ** $p<0.01$. **c)** *Intestinal–type fatty acid binding protein (I–FABP) in the urine of 40 patients undergoing on-pump CABG according to an experimental CPB protocol (n=20) or the standard CPB protocol (n=20). Urinary excretions of I–FABP was calculated as ratio to urine urea concentration in order to correct for urine dilution. The values are represented as mean (symbols) and standard error of the mean (bars).* ** $p<0.01$.

Multivariate regression analysis

The following parameters were examined as potential explanatory variables for the values of postoperative CK–MB, urine NAG and urine I–FABP production: patient age, gender, body surface area, co–morbidities (diabetes type II, preoperative TIA/CVA), perfusion time, cross–clamping time, lowest intraoperative mean arterial pressure, cardiac output, pre/postoperative hematocrit, PC units transfused in the operating room, priming volume, cardioplegic volume (total volume minus recovered volume), lowest core temperature during CPB, administration of peripheral vasoconstrictors.
The postoperative peak of CK–MB correlated at a significance level < 0.2 with the following variables: gender, body surface area, perfusion time, cross–clamping time, postoperative hematocrit, and PC units transfused in the operating room. These variables were introduced in a backward multivariate regression analysis. The best predicting model (R^2=24%, Sig=0.002) included the BSA (B=260.4, Sig.=0.019) and total perfusion time (B=2.001, Sig=0.011).
The postoperative NAG correlated at a significance level < 0.2 with the following variables: gender, body surface area, lowest intraoperative MAP, lowest core temperature during CPB, postoperative hematocrit, PC units transfused in the operating room, administration of peripheral vasoconstrictors, cardioplegic volume and priming volume. These variables were introduced in a backward multivariate regression analysis. The best predicting model (R^2=56.9%, Sig<0.001) included the gender (higher NAG concentrations in women, B=181.1, Sig.=0.009), the postoperative hematocrit (B=–15.19., Sig.=0.054) and PC units transfused in the operating room (B=82.7, Sig=0.065).
The postoperative I–FABP correlated at a significance level < 0.2 with the following variables: age, gender, body surface area, lowest intraoperative MAP, lowest core temperature during CPB, postoperative hematocrit, PC units transfused in the operating room, diabetes, administration of peripheral vasoconstrictors, and priming volume. These variables were introduced in a backward multivariate regression analysis. The best predicting model (R^2=70%, Sig<0.001) included the gender (higher I–FABP concentrations in men, B=-93.4, Sig.=0.048), the postoperative hematocrit (B=–16.19, Sig.=0.022), PC units transfused in the operating room (B=92.18, Sig=0.002), and priming volume (B=0.147, Sig.=0.027).

Univariate regression analysis

No predictors were detected for the variability in postoperative peak CK–MB concentrations in a univariate regression model.
The post–CPB Htc values were significant independent predictors for the variability in postoperative NAG concentrations (adjusted R^2=38.5%, B=–34.87, p<0.001) and I–FABP concentrations (adjusted R^2=37.4%, B=–27.01, p<0.001). Low hematocrit values were significantly associated with high levels of NAG and I–FABP.

Intraoperative PC transfusion was found as significant independent predictor of NAG release (adjusted R^2=44.4%, B=308.2, p<0.001), and I–FABP release (adjusted R^2=31.8%, B=207.1, p<0.001). The patients who received PC transfusion had significantly higher NAG and I–FABP levels.
Priming volume predicted significantly NAG variability (adjusted R^2=13.2%, B=0.236, p=0.012) and I–FABP variability (adjusted R^2=29.3%, B=0.263, p<0.001). A higher priming volume was independently associated with higher urine NAG and I–FABP values.

3.4 Discussion

The data presented in this study documents decreased postoperative myocardial, renal and intestinal tissue injury in patients undergoing on–pump CABG when using a modified operative protocol combining cold crystalloid cardioplegia and intracavitary cooling of the heart, autologous priming, and corporeal normothermia.

Myocardial protection

The protection of the heart during the ischemic arrest time was performed by combining the standard cold crystalloid cardioplegia with an intracavitary cooling system. A cold, sterile saline–filled balloon attached to a dual stage bicaval cannula enabled an additional local, homogeneous cooling of the right atrium. As a consequence, postoperative myocardial damage, as quantified by plasma levels of creatine kinase MB (CK–MB), was significantly lower in the patients in the experimental group.
Increased postoperative peak CK–MB values were demonstrated to be strong predictors of adverse outcomes, indicating increased risk of severe postoperative left ventricular dysfunction and mortality within 30 days of coronary artery bypass grafting[16].

Renal protection

Transient proximal tubules injury was significantly attenuated in the patients benefiting from the experimental operative protocol, as shown by the urine concentrations of N–acetyl–beta–D glucosaminidase (NAG). NAG is a lysosomal enzyme of 130 kDa molecular mass, normally excreted in low amounts in urine as a consequence of the normal exocytosis process[17]. NAG was proposed as a valuable marker of tubulo–interstitial damage in various human glomerular diseases including diabetic nephropathy[18], primary and toxic glomerulonephritis[17,19]. In patients undergoing CPB, measurement of urinary NAG additional to standard clinical tests was demonstrated to be useful in recognizing early and differentiated changes in renal function[20,21].
In our study, NAG levels were in average 4 folds higher in the patients undergoing CPB according to the standard protocol, as compared to the experimental protocol.

Intestinal protection

Transient intestinal damage, as quantified by the urinary excretion of intestinal–type fatty acid binding protein (I–FABP), was significantly decreased in patients undergoing on pump CABG according to the experimental protocol. I–FABP is a 15 kDa cytosolic protein uniquely located in the mature epithelium of the villi, which are most susceptible to ischemic injury [22]. Clinical studies demonstrated that I–FABP is released into the blood stream and excreted by kidneys early in the course of intestinal ischemia [23,24]. Elevated I–FABP urine levels predict the development of gastrointestinal complications after CPB [14], and correlate with the clinical development of the systemic inflammatory response syndrome in critically ill patients [25].
In our study, the urinary excretion of I–FABP in the standard protocol group was in average 2 folds higher than in the experimental protocol group.

Postoperative renal and intestinal injury were independently predicted by three variables: postoperative hematocrit, transfusion requirements during operation, and priming volume of the extracorporeal circuit.

Effect of hematocrit

Analysis of variance for linear regression showed that variation in Htc independently explained 38% and 37% in the variability of postoperative NAG and I–FABP, respectively. A decrease with one unit (1%) in hematocrit predicted significantly an increase with a quarter of the peak postoperative NAG values. The same decrease with one unit (1%) in hematocrit predicted significantly an increase of a tenth of the peak postoperative I–FABP values. In addition, hypoxia during CPB, as quantified by lactate values, correlated positively with intestinal and renal injury postoperative. This association could represent an additional indication that a decrease in hematocrit, with a subsequent decrease in oxygen–carry capacity, might be responsible of intestinal and renal injury post CPB. With regards to the effect of hemodilution on post–operative myocardial injury, our study showed no significant predictor values for the post–operative hematocrit values on the post–operative CK–MB peak concentrations.
These findings quantify for the first time, to our knowledge, the effect of hemodilution and subsequent hematocrit values on postoperative renal and intestinal injury in the clinical setting of on–pump CABG.
These data bring additional information to explain previously reported conclusions of large observational studies reporting a significant greater incidence of postoperative complications as hematocrit values decrease: renal failure ($\sim$ four–fold), multiorgan failure ($\sim$ seven–fold), and septicemia ($\sim$ three–fold) [26].
The combined operative strategy presented in this study allowed higher intra– and post–operative hematocrit values as a consequence of the partial recovery of the car-

dioplegic solution and autologous blood priming of the extracorporeal circuit.

Effect of transfusion requirements

The differences between groups in PC transfusion requirements, both intraoperatively and in the ICU, were significant at a 10% level. Packed cell transfusion in the operating room predicted independently a variation of 44% in NAG values and 32% of I–FABP values. However, in order to subtract from this estimation the effect of low hematocrit of the patients that received transfusion, we introduced in the same regression model both hematocrit values and transfusion requirements. By analyzing the proportions of residuals we could conclude that an extra 21.5% of the variability in NAG and 10% of the variability in I–FABP that could not be explained by the variation in hematocrit values were explained by the transfusion requirements. In other words, intraoperative packed cells transfusion to the patients was correlated significantly with postoperative renal and intestinal injury, independently of the hematocrit value.
The effect described by the present data support the previous raised concerns regarding transfusion–associated morbidity such as hemolytic or allergic reactions, infections (human immunodeficiency virus, cytomegalovirus, hepatitis)[27], graft–versus–host disease[28] and an increased incidence of postoperative infections in patients undergoing coronary artery bypass surgery[29].

Effect of priming volume

The volume of the solution used to prime the extracorporeal circuit was an independent predictor of renal injury (13% of NAG variation) and intestinal injury (29% I–FABP variation). However, this effect proved to reflect only the subsequent hemodilution effect on organ injury.
As a consequence of partial cardioplegic volume recovery, the fluid balance was significantly lower in the experimental group than in the standard group.

Effect of corporeal temperature

The "cold heart, warm body" principle applied in this study proved to be safe and effective in preserving myocardial integrity and, in the same time, in attenuating postoperative intestinal injury. In the hypothermic group, lower intrarectal temperatures measured at 60 min of CPB correlated significantly with higher intestinal damage postoperatively. The association between lower corporeal temperatures with higher postoperative intestinal injury reflects the findings of Ohri and all.[30], showing significantly reduced gastric mucosal blood flow during cross clamping in patients undergoing hypothermic CPB as compared to patients undergoing normothermic CPB. Regarding the validity of normothermic cardiopulmonary bypass, the data presented

in this study support the previous studies evaluating the normothermic CPB associated with topical heart hypothermia, that reported it to be safe[1–5], to simplify surgical procedures, and to facilitate postoperative management[31].
Additional to the description of the independent predictors for renal and intestinal injury, a multivariate analysis revealed the importance of patients demographics, peri–operative variables, medication and co–morbidity for the variability of post operative cardiac, renal and intestinal damage. High postoperative myocardial damage was predicted by a large body surface area and long perfusion durations. Post operative transient renal injury was higher in women, in patients with a low postoperative hematocrit and those who received packed units intraoperatively. Post operative transient intestinal injury was higher in men, in patients with a low postoperative hematocrit, in those patients who had high priming volumes and who received packed units intraoperatively.

3.5 Conclusions

In conclusion, this chapter describes the experimental infrastructure and clinical application of a comprehensive operative strategy that limits postoperative myocardial, renal and intestinal tissue injury in patients undergoing heart–lung machine assisted coronary artery bypass grafting.
The addition of an intracavitary cooling system to the standard cold crystalloid cardioplegia offered a better protection of the myocardium, with decreased postoperative plasma levels of creatine kinase MB. Partial recovery of intra–atrial cardioplegic fluid and autologous blood priming limited the extent of intra–operative hemodilution and blood transfusion requirements (10% significance level), and resulted in an important attenuation of the transient renal and intestinal postoperative injury.

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References

1. Speziale G, Ferroni P, Ruvolo G, Fattouch K, Pulcinelli FM, Lenti L et al. Effect of normothermic versus hypothermic cardiopulmonary bypass on cytokine production and platelet function. *J. Cardiovasc. Surg. (Torino).* 2000;41:819–27.

2. Tonz M, Mihaljevic T, von Segesser LK, Schmid ER, Joller-Jemelka HI, Pei P et al. Normothermia versus hypothermia during cardiopulmonary bypass: a randomized, controlled trial. *Ann. Thorac. Surg.* 1995;59:137–43.

3. Dworschak M, Lassnigg A, Tenze G, Zimpfer D, Czerny M, Grimm M et al. Perfusion temperature during cardiopulmonary bypass does not affect serum S–100beta release. *Thorac. Cardiovasc. Surg.* 2004;52:29–33.

4. Grimm M, Czerny M, Baumer H, Kilo J, Madl C, Kramer L et al. Normothermic cardiopulmonary bypass is beneficial for cognitive brain function after coronary artery bypass grafting–a prospective randomized trial. *Eur. J. Cardiothorac. Surg.* 2000;18:270–5.

5. Grigore AM, Mathew J, Grocott HP, Reves JG, Blumenthal JA, White WD et al. Prospective randomized trial of normothermic versus hypothermic cardiopulmonary bypass on cognitive function after coronary artery bypass graft surgery. *Anesthesiology.* 2001;95:1110–9.

6. Eckmann DM, Bowers S, Stecker M, Cheung AT. Hematocrit, volume expander, temperature, and shear rate effects on blood viscosity. *Anesth. Analg.* 2000;91:539–45.

7. Shin'oka T, Shum-Tim D, Jonas RA, Lidov HG, Laussen PC, Miura T et al. Higher hematocrit improves cerebral outcome after deep hypothermic circulatory arrest. *J. Thorac. Cardiovasc. Surg.* 1996;112:1610-20.

8. Swaminathan M, Phillips-Bute BG, Conlon PJ, Smith PK, Newman MF, Stafford-Smith M. The association of lowest hematocrit during cardiopulmonary bypass with acute renal injury after coronary artery bypass surgery. *Ann. Thorac. Surg.* 2003;76:784–91.

9. Ranucci M, Menicanti L, Frigiola A. Acute renal injury and lowest hematocrit during cardiopulmonary bypass: not only a matter of cellular hypoxemia. *Ann. Thorac. Surg.* 2005;78:1880–1.

10. Morariu AM, Gu YJ, Huet RC, Siemons WA, Rakhorst G, Oeveren WV. Red blood cell aggregation during cardiopulmonary bypass: a pathogenic cofactor in endothelial cell activation? *Eur. J. Cardiothorac. Surg.* 2004;26:939–46.

11. Loef BG, Henning RH, Epema AH, Rietman GW, van Oeveren W, Navis GJ et al. Effect of dexamethasone on perioperative renal function impairment during cardiac surgery with cardiopulmonary bypass. *Br. J. Anaesth.* 2004;93:793–8.

12. Lockwood TD, Bosmann HB. The use of urinary N–acetyl–β–glucosaminidase in human renal toxicology: II. Partial biochemical characterization and excretion in humans and release from the isolated perfused rat kidney. *Toxicol. Appl. Pharmacol.* 1979;49:323–36.

13. Pelsers MM, Namiot Z, Kisielewski W, Namiot A, Januszkiewicz M, Hermens WT et al. Intestinal–type and liver–type fatty acid–binding protein in the intestine. Tissue distribution and clinical utility. *Clin. Biochem.* 2003;36:529–35.

14. Holmes JH 4th, Lieberman JM, Probert CB, Marks WH, Hill ME, Paull DL et al. Elevated intestinal fatty acid binding protein and gastrointestinal complications following cardiopulmonary bypass: a preliminary analysis. *J. Surg. Res.* 2001;100:192–6.

15. Morariu AM, Loef BG, Aarts LPH, Rietman GW, Rakhorst G, van Oeveren W, Epema AH. Dexamethasone: benefit and prejudice for patients undergoing on-pump coronary artery bypass grafting. A study on myocardial, pulmonary, renal, intestinal, and hepatic injury. Accepted to *Chest* 2005

16. Ramsay J, Shernan S, Fitch J, Finnegan P, Todaro T, Filloon T et al. Increased creatine kinase MB level predicts postoperative mortality after cardiac surgery independent of new Q waves. *J. Thorac. Cardiovasc. Surg.* 2005;129:300–6.

17. Price RG. The role of NAG (N-acetyl-beta-D-glucosaminidase) in the diagnosis of kidney disease including the monitoring of nephrotoxicity. *Clin. Nephrol.* 1992;38:S14–9.

18. Rustom R, Costigan M, Shenkin A, Bone JM. Proteinuria and renal tubular damage: urinary N–acetyl–β–D–glucosaminidase and isoenzymes in dissimilar renal disease. *Am. J. Nephrol.* 1998;18:179–185.

19. Bazzi C, Petrini C, Rizza V, Arrigo G, Napodano P, Paparella M et al. Urinary N–acetyl–β–glucosaminidase excretion is a marker of tubular cell dysfunction and a predictor of outcome in primary glomerulonephritis. *Nephrol. Dial Transplant.* 2002;17:1890–6.

20. Dehne MG, Sablotzki A, Muhling J, Dehne KL, Rohrig R, Hempelmann G. Renal effects of cardiopulmonary bypass in the elderly. *Perfusion.* 2002;17:205–9.

21. Loef BG, Epema AH, Navis G, Ebels T, van Oeveren W, Henning RH. Off–pump coronary revascularization attenuates transient renal damage compared with on-pump coronary revascularization. *Chest.* 2002;121:1190–4.

22. Robinson JW, Mirkovitch V. The recovery of function and microcirculation in small intestinal loops following ischaemia. *Gut.* 1972;13:784–9.

23. Gollin G, Marks C, Marks W. Intestinal fatty acid binding protein in serum and urine reflects early ischemic injury to the small bowel. *Surgery.* 1993;113:545–51.

24. Lieberman JM, Sacchettini J, Marks C, Marks WH. Human intestinal fatty acid binding protein: report of an assay with studies in normal volunteers and intestinal ischemia. *Surgery.* 1997;121:335–42.

25. Lieberman JM, Marks WH, Cohn S, Jaicks R, Woode L, Sacchettini J et al. Organ failure, infection, and the systemic inflammatory response syndrome are associated with elevated levels of urinary intestinal fatty acid binding protein: study of 100 consecutive patients in a surgical intensive care unit. *J. Trauma.* 1998;45:900–6.

26. Habib RH, Zacharias A, Schwann TA, Riordan CJ, Durham SJ, Shah A. Adverse effects of low hematocrit during cardiopulmonary bypass in the adult: should current practice be changed? *J. Thorac. Cardiovasc. Surg.* 2003;125:1438–50.

27. Goodnough LT, Brecher ME, Kanter MH, AuBuchon JP. Transfusion medicine. Blood transfusion (first of two parts). *N. Engl. J. Med.* 1999;340:438–447.

28. Thaler M, Shamiss A, Orgad S, Huszar M, Nussinovitch N, Meisel S et al. The role of blood from HLA–homozygous donors in fatal transfusion–associated graft–versus–host disease after open–heart surgery. *N. Engl. J. Med.* 1989;321:25–8.

29. Chelemer SB, Prato BS, Cox PM Jr, O'Connor GT, Morton JR. Association of bacterial infection and red blood cell transfusion after coronary artery bypass surgery. *Ann. Thorac. Surg.* 2002;73:138–42.

30. Ohri SK, Bowles CW, Mathie RT, Lawrence DR, Keogh BE, Taylor KM. Effect of cardiopulmonary bypass perfusion protocols on gut tissue oxygenation and blood flow. *Ann. Thorac. Surg.* 1997;64:163–70.

31. Nappi G, Torella M, Romano G. Clinical evaluation of normothermic cardiopulmonary bypass and cold cardioplegia. *J. Cardiovasc. Surg. (Torino)* 2002;43:31–6.

Chapter 4

Organ Perfusion During Cardiopulmonary Bypass: Vascular Endothelium Activation.

Red Blood Cell Aggregation During Cardiopulmonary Bypass: A Pathogenic Cofactor in Endothelial Cell Activation?

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Abstract

Introduction

The bio–incompatibility of the cardiopulmonary bypass (CPB) circuit and the use of artificial colloids trigger massive defense reactions that involve endothelial cells and several blood cells: platelets, neutrophils, monocytes, red blood cells (RBC) and lymphocytes.
Investigating the effects on RBC aggregation and endothelial cells activation, the present study addresses two different prime solutions commonly used in clinical practice.

Patients, Materials and Methods

RBC aggregation was measured by means of Laser–assisted Optical Rotation Cell Analyzer, in an in vitro study designed to mimic human blood–material interactions during extracorporeal circulation.
A clinical study investigating endothelial activation was conducted in 20 patients undergoing elective coronary bypass surgery, randomly assigned for CPB using two different priming solutions: HAES–steril 6% (HES 200/0.5) and Voluven 6% (HES 130/0.4).

Results

Circulation trough a Chandler loop of HES–blood mixtures altered significantly RBC aggregability. The use of HES 130/0.4 resulted in marked decrease in RBC aggregation (Aggregation Index AI before and after circulation 23.5±3.8 and 18±2.9, respectively), no significant differences being found when compared with Ringer's lactate group. The use of HES 200/0.5 resulted in better maintained RBC aggregation (AI 39.7±5.9 and 29.7±4.7 before and after circulation, respectively). The AI measured for the whole blood (control) sample was 61.9±4.9 before circulation, and 58.1±4 after.
Markers of endothelial activation (von Willebrand factor–vWF, thrombomodulin–TM, tissue Plasminogen Activator–tPA and E–selectin) significantly increased during CPB. Differences between HES treatment groups were evident post–bypass. While the markers of endothelial activation returned to baseline in HES 200/0.5 group, HES 130/0.4 was associated on the first postoperative day with further increase of vWF and tPA.

Conclusions

RBC aggregation significantly drooped as consequence of blood dilution and blood–

material interaction. We reason that low RBC aggregation added to plasma viscosity reduction and non–physiologic flow conditions during extracorporeal circulation, are important factors contributing to variations in shear stress at the venous endothelial wall. The variations in shear stress might trigger complex signaling leading to endothelial activation. Additional fundamental research is needed in order to verify the hypothesis introduced by the present study.
Characterizing the impact of rheologic parameters on endothelial function could prove to be valuable in patients undergoing CPB.

4.1 Introduction

Cardiac surgery involving cardiopulmonary bypass (CPB) leads to activation of the haemostatic–inflammatory systems associated with increased postoperative morbidity and prolonged hospital stay[1]. As documented in the literature, extracorporeal circulation triggers massive defense reactions that involve endothelial cells and at least five blood cells: platelets, neutrophils, monocytes, red blood cells and lymphocytes. The intensity of the cellular and humoral activation is known to vary with patient factors, the surface area of biomaterials, duration of perfusion and exposure of blood to the wound[2]. As a consequence, a whole–body inflammatory response and microemboli are generated, leading to fever and leukocytosis, compromised fluid balance, ischemia and microinfarctions[3].

The relations between a low hematocrit and adverse outcomes in patients undergoing CPB is extensively discussed in the literature[4]. There are also reports addressing the mechanical trauma in red blood cells[5] and the decrease in red blood cell deformability during extracorporeal circulation[6].

In our opinion, a complete chapter has been excluded from the discussion around the pathogenesis of the "post–CPB syndrome": modifications induced in red blood cell aggregation and potential consequences on microcirculation.

The aggregation property of red blood cells is mainly considered to be pathophysiologic, since aggregation is elevated in many disease states such as diabetes mellitus[7] and hypertension[8]. However, red cell aggregation is normally present in humans and other "athletic" species being most pronounced in those species having the highest capacity for oxygen consumption, while it is absent in sedentary animals[9]. This raised the possibility that normal levels of aggregation may serve homeostasis, having functional significance for normal physiology[10]. Earlier experiments conducted in our group conclusively showed that the physiological function of red blood cells to form aggregates is significantly affected in the presence of hydroxyethyl starch (HES)[11]. Since HES solutions are extensively used as volume substitutes and priming solutions, RBC aggregation is expected to suffer deviation from normal values during cardiopulmonary bypass. Additionally, the unavoidable hemodilution associated with the use of the heart–lung machine is expected to result also in a drop of plasma viscosity. These hemodynamic alterations could represent mechanical triggers of further endothelial cell activation already exposed to other insults occurring during CPB, such as hypoxia, inflammatory stimuli, and surgical manipulation. Endothelial activation is known to disrupt the barrier function, enhance vasoconstriction and increase the leukocyte adhesion[12].

The present study aims to test the potential effect of blood interactions with HES solutions and extracorporeal circuit on red blood cells aggregability and to document endothelial cell activation in the presence of two different prime solutions commonly used in the clinical practice, HAES–steril 6% and Voluven 6%. The clinical relevance and possible correlation between the pathophysiological mechanisms implicated are

discussed.

4.2 Patients, Materials and Methods

Red Blood Cell Aggregability, Blood Viscosity and Plasma Viscosity

RBC aggregability was investigated in vitro with a Laser–assisted Optical Rotation Cell Analyzer (LORCA R&R Mechatronics, Hoorn, The Netherlands). This instrument, based on the ektacytometric principle, is equipped with a video camera for detection of the laser diffraction–pattern, a thermostation unit and an ellipse–fit computer program calculating the Aggregation Index (AI) of RBCs[13]. For the determination of red cell aggregation, the blood is brought under a shear rate of $500\,s^{-1}$, after which the shear is stopped (t=0). The backscattered intensity from the blood layer is measured during 120 s after shear stop. The intensity drops because of red blood cell aggregation. We considered the beginning point of the aggregation the extrapolated value of the decay curve towards t=0[14].
The RBC aggregation measurements were performed at 32° C, in samples prepared by in vitro admixture of HES solutions (either HAES–steril 6% or Voluven 6%) to human fresh blood from healthy volunteers (n=6), drawn from the antecubital vein, heparinized (4 U/ml) and oxygenated (10 min). The blood:prime mixture ratio was 5:2 by volume. In order to asses the effect of dilution alone, we measured also the RBC aggregation in Ringer's Lactate (RL)–blood mixtures (blood:RL ratio = 5:2). We considered as controls AI values measured in whole blood samples.
AI was measured before and after sample circulation trough a Chandler loop[15] of silicon tubing, mimicking the blood–material/device interactions during extracorporeal circulation. Silicon tubing with a total volume of 6.3 ml (inner diameter 4 mm, length 500 mm) was filled with 4.5 ml of sample leaving a gas volume of 1.8 ml. The tubing was closed into a loop using PVC connectors and then circulated vertically at 10 rpm, in a 32° C water bath for one hour.
The choice of using the Chandler loop as a model was based on results of numerous in vitro studies comparing the use of simpler or more complex in vitro models for characterization of blood–material/device interactions. Coagulation parameters, platelets activity and hematolysis were monitored in each model. In this regard, testing in the simple Chandler loop model produced findings, which overlapped with observations from the more complex CPB models[16].
Viscosity was measured by means of an automated dynamic shear rheometer with cone–plane geometry (AR1000 Rheometer, TA Instruments). Viscosity was measured both in blood–HES samples and plasma–HES samples. The blood–HES samples were prepared using the same method as used for the RBC aggregation measurements. The plasma–HES samples were prepared by in vitro admixture of HES solution to human fresh plasma in a mixture ratio of 5:2. In order to measure the modification induced by the dilution alone, we also measured viscosity in plasma samples mixed

with RL in the same ratio. During viscosity measurements the temperature was set at 32° C. Viscosity was measured at four shear rates for blood samples ($30\,s^{-1}$, $60\,s^{-1}$, $100\,s^{-1}$ and $200\,s^{-1}$) and at three shear rates for plasma samples ($60\,s^{-1}$, $100\,s^{-1}$ and $200\,s^{-1}$).

Endothelial activation during CPB

A prospective randomized single blind study, approved by the Medical Ethics Committee of Hospital de Weezenlanden in Zwolle, Netherlands, was conducted in 20 patients, who underwent an elective coronary bypass surgery. The patients were randomly assigned for cardiopulmonary bypass with either HAES–steril 6% or Voluven 6%.
The patients were less than 75 years of age, had a body weight over 65 kg, underwent a cardiopulmonary bypass time of more than 30 minutes and had signed a written consent. Exclusion criteria were presence of severe heart failure, renal or liver dysfunction, bleeding diathesis, diabetes mellitus, and the use of platelet inhibiting drugs within five days before the operation.
Induction and maintenance of anesthesia, surgical techniques and cardiopulmonary bypass procedures including anticoagulation with heparin and its neutralization with protamine, were performed in a standardized fashion [17].
The extracorporeal circuit consisted of an integrated microporous plate membrane oxygenator (Cobe–Duo, Cobe, CO, Lakewood, USA), polyvinyl chloride tubing and a centrifugal pump (Biomedicus, Medtronic, Anaheim, CA, USA). The priming volume of the circuit was two liters and the priming solution compositions were:

- HAES–steril 6% (Fresenius AG, Oberursel, Germany) 1000 ml (6% HES 200/0.5, median molecular weight 200 kD, degree of substitution 0.5) supplemented with Ringer's lactate to a final concentration of 3%.

- Voluven 6% (Fresenius AG, Oberursel, Germany) 1000 ml (6% HES 130/0.4, median molecular weight 130 kD, degree of substitution 0.4) supplemented with Ringer's lactate to a final concentration of 3%.

HES solutions served also as plasma substitutes, the dose limitation being 3 litters in the pre–, during, and post–operative period. After reaching these defined study colloid dose limits postoperatively, isotonic pasteurized plasma was administered in case that additional volume was needed. As standard practice in our clinic, 1500 IU heparin was added to all priming solutions.
During the operative day and on the first postoperative day, three blood samples were taken for biochemical determinations: after induction of anesthesia (post–induction), at arrival on the intensive care unit (1 h ICU), and on the first postoperative day (1^{st} POD). The post–CPB values (1 h ICU) were corrected for plasma dilution using hemoglobin values.

Blood samples were obtained from the radial artery catheter and were mixed with 3.06% sodium citrate, with a volume ratio of 9:1. The samples were kept on ice during storage. The citrated blood was centrifuged at 1100 g for 12 minutes to obtain platelet poor plasma, stored at –80° C until further determinations of biochemical assays. Plasma concentrations of endothelial and/or platelet release products were investigated by means of ELISA: von Willebrand Factor (vWF) (Gradipore, North Ride, Australia); tissue plasminogen activator (t–PA) (Coaliza, Innogenetics, Belgium); E– and P–Selectine (R&D Systems, inc, Abingdon,UK); thrombomodulin (TM) (Imubind, American Diagnostica inc, Greenwich, CT, USA).

Statistical analysis

Before data analysis, all individual sample points were tested for distribution according to the Kolmogorov–Smirnov goodness of fit test. In case of not normally distributed data, Mann–Whitney test was used to quantify differences between groups. Within groups Wilcoxon Signed Ranks test was performed to show differences during treatment. Correlations between variables was tested using Spearman's correlation test.
To detect possible differences in effect of each priming solution, for normal distributed data, one way analysis of variance (ANOVA) was used to compare groups. If differences between the groups were significant ($p<0.05$), post hoc multiple comparisons were performed to quantify any differences among groups using the Tukey HSD test with a level of significance $p<0.05$. A Bonferroni correction was made for multiple testing. Within groups a paired T–test was performed to show differences during treatment.
The variables are expressed as mean±SEM, unless stated otherwise.

4.3 Results

Red Blood Cell Aggregation

The AI measured in control samples was 61.9±4.9 before circulation, and 58.1±4 after. Dilution of blood with Ringer's lactate solution yielded a decrease of AI to 16.6±2.6. Mixture with HES 130/0.4 resulted in low aggregation (AI before and after circulation 23.5±3.8 and 18±2.9, respectively). No significant differences were found between Ringer's lactate group and HES 130/0.4 group. The use of HES 200/0.5 compensated by half the dilution effect on red blood cell aggregation; AI values in this group were 39.7±5.9 and 29.7±4.7 before and after circulation, respectively (Fig. 4.1). Circulation trough the closed silicone tubing system of blood: HES mixture significantly reduced red blood cell aggregability (paired Student Test $p\leq0.01$).

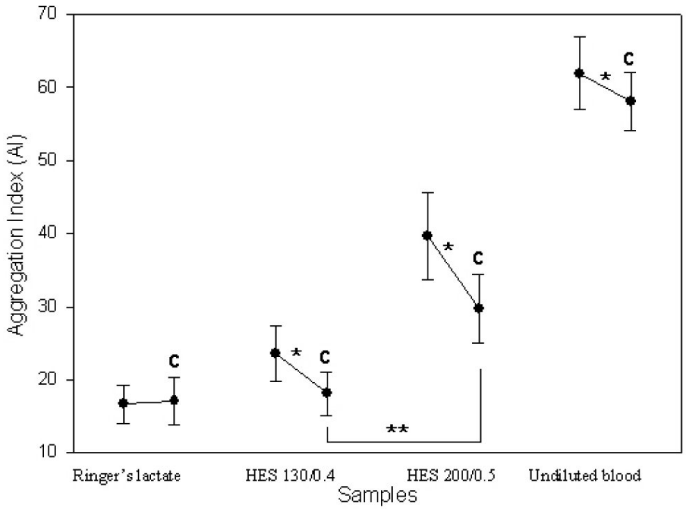

Figure 4.1: Red blood cell aggregation index measured before and after circulation (C) through a closed silicon tubing in 4 groups of blood treated samples (mixing ratio 5:2): (i) Ringer's lactate; (ii) HES 130/0.4; (iii) HES 200/0.5; (iv) Control. The values are represented as mean (symbols) and standard deviation of the mean (bars). Significant ($p<0.05$) and highly significant differences ($p<0.01$) within and between the groups are indicated with * and **, respectively.

Method validation for AI measurements

Measurements of Normal Reference sample showed a mean value of 57.79, a SD of 0.88, a 95% CI of [56.86; 58.72] and a coefficient of variation of 14.66%. Measurements of Low Reference sample (blood:RL=5:2) showed a mean value of 16.72, a SD of 1.49, a 95% CI of [15.15; 18.29] and a coefficient of variation of 24.83. The AI was measured 6 consecutive times at 32° C.

Blood viscosity

Fig. 4.2a shows the viscosity curve of blood samples measured at 32° C and shear rates $30\,s^{-1}$, $60\,s^{-1}$, $100\,s^{-1}$ and $200\,s^{-1}$. When measuring at a shear rate of $30\,s^{-1}$, the lowest values were registered after mixture with Ringer's lactate solution (drop to 59% of whole blood viscosity), followed by values given by HES 130/0.4 group (drop to 64% of control values) and HES 200/0.5 (drop to 72% of control values). ANOVA showed significant differences between groups ($p<0.001$). Multiple comparison with post–hoc test pointed out that differences between the groups were significant at all tested shear rates ($p<0.001$). The same ranking was observed when measuring plasma

and plasma–HES mixtures viscosities (Fig. 4.2b). When measuring at a shear rate of $100\,s^{-1}$, the addition of RL, HES 130/0.4 and HES 200/0.5 determined a decrease in plasma viscosity to 75%, 85% and 92% of the initial values, respectively. ANOVA showed significant differences between the groups. Post–hoc tests demonstrated that the values between the groups were significantly different at all measured shear rates ($p<0.001$), excepting the differences at a shear rate of $60\,s^{-1}$ between Plasma:RL and Plasma:HES 130/0.4 groups ($p=0.137$).

Endothelial activation during CPB

No significant differences were measured between the two groups with regard to age, sex, weight, body surface area, cardiopulmonary bypass and aortic cross clamp time, number and origin of grafts or volumes infused. Also, no relevant differences were found in concomitant diseases or medication between the treatment groups. On the intensive care unit, 40% of the patients from both HES 130/0.4 and HES 200/0.5 groups received additional isotonic pasteurized plasma, 178 ml in average, after the dose limit of three liters colloid was reached. 40% patients in the HES 130/0.4 group and 50% patients in HES 200/0.5 group received allogenic blood products.

Von Willebrand Factor (plasma) concentration did not change significantly during extracorporeal circulation, although in both groups a trend to increase was observed. Between induction of anesthesia and the end of the surgical procedure vWF concentrations ranged between 60 and 260% of normal pooled plasma, being in average higher than normal. The values in HES 130/0.4 group started to increase in the reperfusion period; the concentrations in the first postoperative day were significantly higher than baseline (Wilcoxon Sig.<0.01). Significant differences were measured between groups, with higher plasma values in HES 130/0.4 group (Mann–Whitney Sig.≤ 0.01) (Fig. 4.3).

Tissue–Plasminogen Activator concentrations were significantly higher at 1 h ICU in comparison with baseline values (Wilcoxon Sig.≤ 0.05). The values measured in HES 200/0.5 group were significantly higher than the values in HES 130/0.4 group (Mann-Whitney Sig.<0.01). During reperfusion time, the values in HES 200/0.5 group declined while the values in HES 130/0.4 group increased further (Fig. 4.4).

Thrombomodulin values significantly increased after the end of extracorporeal circulation (Wilcoxon Sig.<0.05) for patients of both groups, with no difference between treatment groups. The values went down in the reperfusion period but remained significantly above the baseline (Wilcoxon Sig.<0.01) (Fig. 4.5).

E–Selectin increased moderately but significantly after CPB in both groups (Wilcoxon Sig.≤ 0.01) with no differences between them. During reperfusion time the values reached baseline levels (Fig. 4.6).

P–Selectin did not change significantly in either group, at any time point (data not shown).

Correlations: in the HES 130/0.4 treatment group vWF values correlated positively

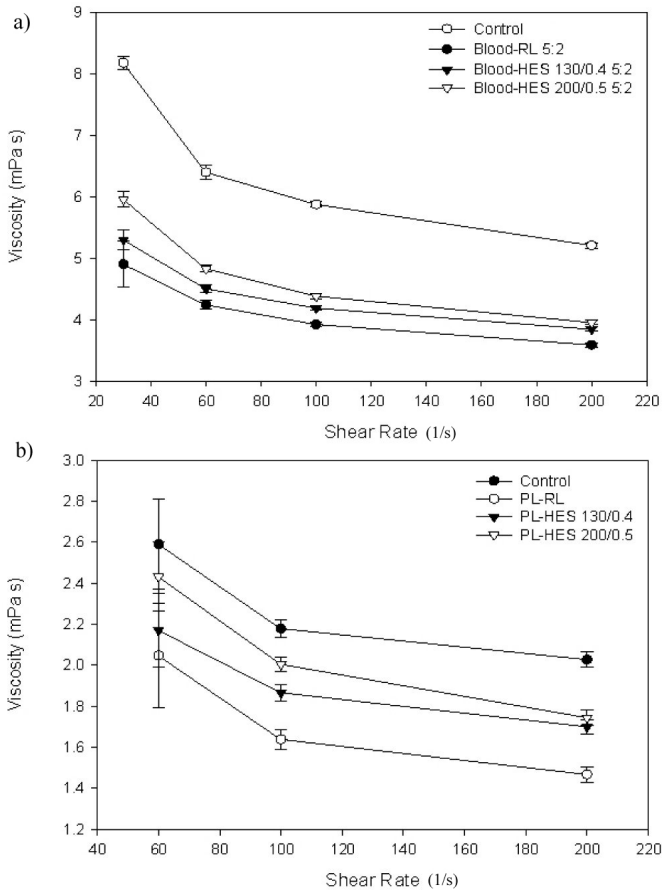

Figure 4.2: In vitro viscosity (mPa.s) of treated blood **(a)** *and plasma* **(b)** *samples (mixing ratio 5:2) measured in 4 groups: (i) Blood/Plasma; (ii) Blood/PL–RL: Blood/Plasma treated with Ringer's lactate; (iii) Blood/PL–HES 130/0.4: Blood/Plasma treated with HES 130/0.4; (iv) Blood/PL–HES 200/0.5: Blood/Plasma treated with HES 200/0.5. The values are represented as mean (symbols) and standard deviation of the mean (bars).*

with the tPA values (Spearman's coefficient 0.681, sig.<0.001). In the HES 200/0.5 group a positive correlation was found between TM and tPA (Spearman's correlation 0.445, sig.=0.016).

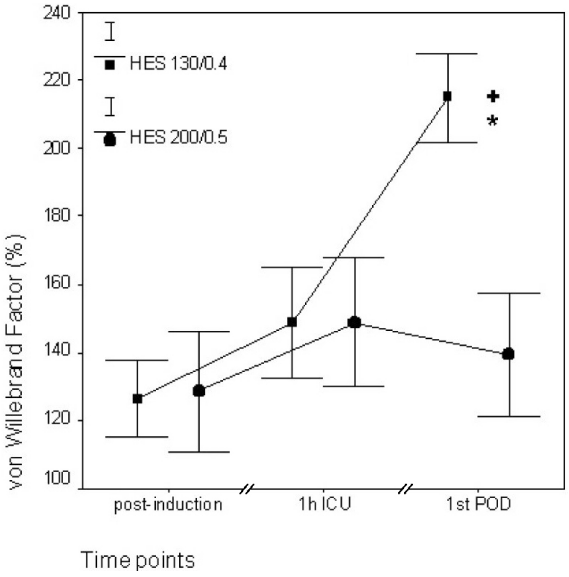

Figure 4.3: von Willebrand factor (vWF) plasma concentration (%) measured in 2 groups of patients: (i) group HES 130/0.4; (ii) group HES 200/0.5. The measurements were effectuated at 3 time points: post induction of anesthesia (post–induction), 1 h after transfer in intensive care unit (1 h ICU) and in the first postoperative day (1st POD). The values are represented as mean (symbols) and standard error of the mean (bars). ⋆ Significant increase between groups ($p<0.01$). $^{+}$ Significant increase compared to baseline ($p<0.01$).

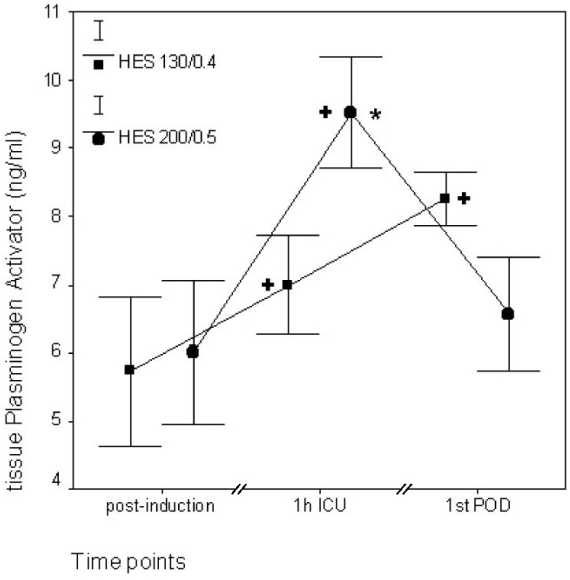

Figure 4.4: Tissue Plasminogen Activator (tPA) plasma concentration (ng/ml) measured in 2 groups of patients: (i) group HES 130/0.4; (ii) HES 200/0.5. The measurements were effectuated at 3 time points (see legend Fig. 4.3). The values are represented as mean (symbols) and standard error of the mean (bars). ⋆ Significant increase between groups ($p<0.01$). $^{+}$ Significant increase compared to baseline ($p<0.05$).

Circulating platelet count

The mean platelet number was comparable between the treatment groups within the evaluation period. The platelet count remained within normal ranges except for

the initial period after CPB (1 h ICU) when values decreased due to dilution effect. Corrected values showed no significant differences as compared to baseline values.

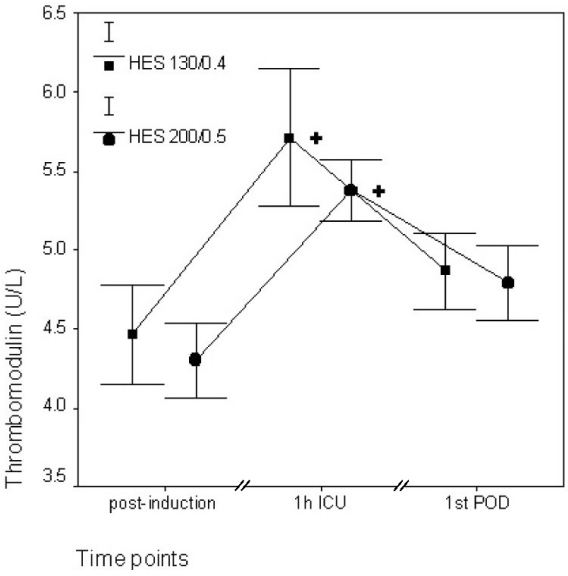

Figure 4.5: Thrombomodulin (TM) plasma concentration (U/L) measured in 2 groups of patients: (i) group HES 130/0.4; (ii) HES 200/0.5. The measurements were effectuated at 3 time points (see legend Fig. 4.3). The values are represented as mean (symbols) and standard error of the mean (bars). $^{+}$ *Significant increase compared to baseline ($p<0.01$).*

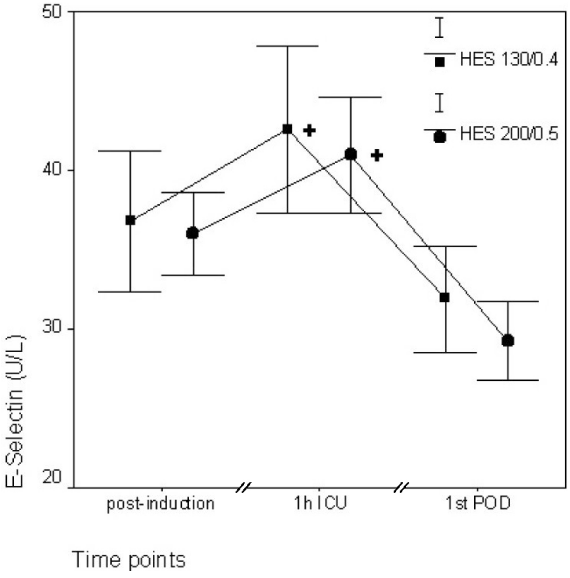

Figure 4.6: E–Selectin plasma concentration (U/L) measured in 2 groups of patients: (i) group HES 130/0.4; (ii) HES 200/0.5. The measurements were effectuated at 3 time points (see legend Fig. 4.3). The values are represented as mean (symbols) and standard error of the mean (bars). $^{+}$ *Significant increase compared to baseline ($p<0.01$).*

4.4 Discussion

The property of red blood cells to form aggregates at low shear rates was profoundly altered in our in vitro model mimicking the human blood–material interactions during extracorporeal circulation. In Ringer's lactate and HES 130/0.4 treated blood samples, the aggregation index (AI) dropped to a quarter of the control AI values. Further decrease was registered as a consequence of blood circulation through silicon tubing. The use of HES 200/0.5 compensated by half the dilution effect on red blood cell aggregation.

In parallel with the decrease in red blood cell aggregation, blood viscosity declined also. The highest viscosity was measured in HES 200/0.5 treated blood samples, followed by HES 130/0.4 and Ringer's lactate treated blood samples. The same ranking was observed when measuring the viscosity of plasma samples.

Current understanding of the rheological effects of red blood cell aggregation suggests that blood shear stress at the venular wall varies when RBC aggregability varies[10,18]. Accordingly, because of RBC aggregation drop, plasma viscosity reduction and non–physiologic flow conditions, it is expected that the blood shear stress at the venous endothelial wall would alter during extracorporeal circulation.

Endothelial cells are notorious for their ability to sense variations in mechanical forces, such as shear stress. Endothelial cells in vivo are normally exposed and presumably adapted to a normal level of shear stress in the range of 0.5–2 Pa. Cells adapted to flow might be expected to respond to either an increase or decrease in shear stress from the normal level. Studies investigating the response of flow–adapted endothelial cells to an abrupt variation in shear stress, showed membrane depolarization, increased intracellular Ca^{2+}, nitric oxide and reactive oxygen species generation[19]. In addition to synthesis and release on demand, several stored compounds are secreted during endothelial cell stimulation, in a Ca^{2+} dependent way. Elevation in intracellular Ca^{2+} triggers release of several vasoactive factors and factors involved in hemostasis and thrombolysis: nitric oxide, prostacyclins, vWF, tPA, tissue factor, adhesion molecules and chemoattractant proteins[20]. In our clinical study vWF and tPA recovered in HES 200/0.5 group while further increasing in the HES 130/0 group.

Because of lack of consensus in literature over the "gold standard" for endothelial activation, our clinical study was designed to measure several markers related to endothelial activation: vWF, TM, t–PA, E–Selectin.

von Willebrand factor is a component of platelet–granules and Weibel–Palade bodies in the endothelial cells. The majority of plasma vWF is derived from endothelial cells and an increase in plasma levels is generally considered to be mainly a marker of endothelial activation. However, vWF is also known to be an acute phase reactant affected by inflammatory cytokines, and as such, may be elevated even in the absence of definite endothelial damage[21,22].

Thrombomodulin is a surface protein of endothelial cells, which acts as a thrombin receptor and serves as an anticoagulation factor. Soluble fragments of TM, proba-

bly components of degradation, circulate in plasma. TM is not released in plasma constitutively or as a response to endothelial activation, but is released after acute endothelial cell injury. As a drawback, TNF–α leads to a reduction in thrombomodulin expression by endothelial cells[22].
Endothelial release of tissue Plasminogen Activator initiates fibrinolysis. tPA may be used to evaluate endothelial stimulation induced by CPB, denoting a postischemic antithrombotic function of the endothelium[23].
E– and P–selectins belong to the selectin family of adhesion molecules and both have been reported to increase in circulation or at lesion sites of several diseases reflecting endothelial activation. The disadvantage of using E–selectin as a marker is the fact that, E–selectin being an leukocyte adhesion molecule, some may be bound to its ligand in vivo, and be unavailable for measurement[22].

The findings of this study showed functional and/or structural alteration of vascular endothelial cell during extracorporeal circulation, as documented by elevated plasma concentrations of vWF, thrombomodulin, tPA and E–selectin. These markers have a proven endothelial origin, since platelet count was similar in both groups and didn't vary extensively during CPB. In the HES 130/0.4 treatment group the increase in vWF correlated positively with the increase in tPA. In the HES 200/0.5 group a positive correlation was found between TM and tPA.
Differences between HES groups were evident post–bypass. While the markers of endothelial activation recovered in HES 200/0.5 group, HES 130/0.4 was associated on the first postoperative day with further increase of vWF and tPA.
These reports may prove to represent additional help in the decision process of the clinician who is confronted with cardiac patients of different etiologies. Even if further documentation is needed, our results documenting the important rise in von Willebrand factor suggest the necessity of a more careful selection of HES solutions. Hypertensive and atherosclerotic patients who have high basal levels of vWF might benefit from HES 200/0.5. HES 130/0.4 could represent a first choice for patients with bleeding tendencies and patients with acquired von Willebrand syndrome after aortic stenosis. In this respect, HES 130/0.4 was proved to be in various clinical settings at least comparable or better on coagulation parameters, blood loss or blood product consumption as compared to HES 200/0.5[17].
Our observations made in vitro on RBC aggregability coupled to the observation made in vivo on endothelial cell activation suggest a hypothetical new pathophysiological mechanism implicated in the post–CPB syndrome. We hypothesize that the drop in RBC aggregation added to plasma viscosity reduction and non–physiologic flow conditions during extracorporeal circulation, are important factors contributing to variation in shear stress at the venous endothelial wall. The variation in shear is known to lead to a complex signaling response eventuating in membrane depolarization, intracellular Ca^{2+} accumulation with subsequent release of nitric oxide, prostacyclins,

vWF, tPA, tissue factor, and generation of reactive oxygen species.
Additional fundamental research is needed in order to verify the hypothesis introduced by the present study. Characterization of the interrelation between rheologic parameters and endothelial function could prove to be valuable in managing complications in CPB patients.

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References

1. Roach GW, Kanchuger M, Mangano CM, Newman M, Nussmeier N, Wolman R, Aggarwal A, Marschall K, Graham SH, Ley C. Adverse cerebral outcomes after coronary bypass surgery. Multicenter Study of Perioperative Ischemia Research Group and the Ischemia Research and Education Foundation Investigators. *N. Engl. J. Med.* 1996;335:1857–63.

2. Edmunds LH Jr. The evolution of cardiopulmonary bypass: lessons to be learned. *Perfusion.* 2002;17:243–51.

3. Ascione R, Lloyd CT, Underwood MJ, Lotto AA, Pitsis AA, Angelini GD. Inflammatory response after coronary revascularization with or without cardiopulmonary bypass. *Ann. Thorac. Surg.* 2000;69:1198–204.

4. Groom RC. High or low hematocrits during cardiopulmonary bypass for patients undergoing coronary artery bypass graft surgery? An evidence-based approach to the question. *Perfusion.* 2002;17:99–102.

5. Svenmarker S, Jansson E, Stenlund H, Engstrom KG. Red blood cell trauma during cardiopulmonary bypass: narrow pore filterability versus free haemoglobin. *Perfusion.* 2000;15:33–40.

6. Kameneva MV, Undar A, Antaki JF, Watach MJ, Calhoon JH, Borovetz HS. Decrease in red blood cell deformability caused by hypothermia, hemodilution, and mechanical stress: factors related to cardiopulmonary bypass. *ASAIO J.* 1999;45:307–10.

7. Martinez M, Vaya A, Server R, Gilsanz A, Aznar J. Alterations in erythrocyte aggregability in diabetics: the influence of plasmatic fibrinogen and phospholipids of the red blood cell membrane. *Clin. Hemorheol. Microcirc.* 1998;18:253–8.

8. Cicco G, Pirrelli A. Red blood cell (RBC) deformability, RBC aggregability and tissue oxygenation in hypertension. *Clin. Hemorheol. Microcirc.* 1999;21:169–77.

9. Popel AS, Johnson PC, Kameneva MV, Wild MA. Capacity for red blood cell aggregation is higher in athletic mammalian species than in sedentary species. *J. Appl. Physiol.* 1994;77:1790–4.

10. Bishop JJ, Popel AS, Intaglietta M, Johnson PC. Rheological effects of red blood cell aggregation in the venous network: a review of recent studies. *Biorheology.* 2001;38:263–74.

11. Morariu AM, Vd Plaats A, V Oeveren W, 'T Hart NA, Leuvenink HG, Graaff R, Ploeg RJ, Rakhorst G. Hyperaggregating effect of hydroxyethyl starch components and University of Wisconsin solution on human red blood cells: a risk of impaired graft perfusion in organ procurement? *Transplantation.* 2003;76:37–43.

12. Verrier ED, Morgan EN. Endothelial response to Cardiopulmonary Bypass Surgery. *Ann. Thorac. Surg.* 1998;66:S17–9.

13. Hardeman MR, Dobbe JG, Ince C. The Laser-assisted Optical Rotational Cell Analyzer (LORCA) as red blood cell aggregometer. *Clin. Hemorheol. Microcirc.* 2001;25:1–11.

14. Graaff R, Gu YJ, Boonstra PW, van Oeveren W, Rakhorst G. Analysis of red blood cell aggregation in cardio-pulmonary bypass (CPB) surgery. *Int. J. Artif. Organs.* 2004;27:488–94.

15. Chandler AB. In vitro thrombotic coagulation of blood. *Lab. Invest.* 1958;7:110–114.

16. Munch K, Wolf MF, Gruffaz P, Ottenwaelter C, Bergan M, Schroeder P, Fogt EJ. Use of simple and complex in vitro models for multiparameter characterization of human blood-material/device interactions. *J. Biomater. Sci. Polym. Ed.* 2000;11:1147–63.

17. Gallandat Huet RC, Siemons AW, Baus D, van Rooyen-Butijn WT, Haagenaars JA, van Oeveren W, Bepperling F. A novel hydroxyethyl starch (Voluven) for effective perioperative plasma volume substitution in cardiac surgery. *Can. J. Anaesth.* 2000;47:1207–15.

18. Bishop JJ, Nance PR, Popel AS, Intaglietta M, Johnson PC. Effect of erythrocyte aggregation on velocity profiles in venules. *Am. J. Physiol. Heart Circ. Physiol.* 2001;280:H222–36

19. Fisher AB, Chien S, Barakat AI, Nerem RM. Endothelial cellular response to altered shear stress. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2001;281:L529–33.

20. Nilius B, Droogmans G. Ion Channels and their functional role in vascular endothelium. *Physiological Reviews.* 2001;4:1415–9.

21. Tsang GM, Allen S, Pagano D, Wong C, Graham TR, Bonser RS. Von Willebrand factor and urinary albumin excretion are possible indicators of endothelial dysfunction in cardiopulmonary bypass. *Eur. J. Cardiothorac. Surg.* 1998;13:385–91.

22. Chong AY, Blann AD, Lip GY. Assessment of endothelial damage and dysfunction: observations in relation to heart failure. *QJM.* 2003;96:253–67.

23. Valen G, Eriksson E, Risberg B, Vaage J. Fibrinolysis during cardiac surgery. Release of tissue plasminogen activator in arterial and coronary sinus blood. *Eur. J. Cardiothorac. Surg.* 1994;8:324–30.

Chapter 5

Organ Perfusion During Cardiopulmonary Bypass: Blood Rheology and Endothelial (Dys)function.

Acute Isovolemic Hemodilution Triggers Pro–Inflammatory and Pro–Coagulatory Endothelial Activation in Vital Organs: Role of Erythrocytes Aggregation

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Abstract

The essential role of erythrocytes as oxygen carriers is historically well established, however their function to aggregate with consequences on homeostasis is under debate. The pathogenic potential of low erythrocyte aggregation might have implications for patients undergoing on–pump cardiopulmonary bypass who are severely hemodiluted due to preoperative isovolemic hemodilution (IHD), circuit priming, and large fluid infusions peri–operatively. Considering the vascular endothelium sensitivity to variations in blood rheology, we hypothesize that low erythrocyte aggregation will be responsible for activation of vascular endothelium during acute IHD. To verify this theory, we induced acute IHD (30 ml/kg exchange–transfusion with colloid–solutions) in an "aggregating species" (pigs, n=15), and investigated the hypoxic oxidative stress (plasma Malondialdehyde, ex–vivo oxygen radicals production in heart, lung, kidney, liver, ileum tissue biopsies), erythrocyte aggregation (LORCA), and endothelial activation (Real Time Quantitative RT–PCR to analyze von Willebrand Factor (vWF), E– and P–Selectins, endothelial nitric oxide synthase gene–expression in tissue biopsies). The production of superoxide and hydroxyl radicals, measured as H_2O_2 generation, was similar at all times in sham–operated and hemodiluted animals, proving a maintained oxygen delivery to tissues. Acute IHD was followed by a dramatic drop in erythrocyte aggregation and immediate pro–thrombotic (significant vWF mRNA up–regulation in heart, lungs, kidney, liver, ileum) and pro–inflammatory (significant E– and P–Selectins mRNA up–regulation in lungs and ileum) endothelial activation. Low erythrocyte aggregation was statistically significantly correlated with increased mRNA–expression of vWF (heart, liver, ileum) and P–Selectin (lungs, ileum and heart). These results suggest that low erythrocyte aggregation can actively trigger endothelium–dependent thrombogenic and pro–inflammatory response during acute isovolemic hemodilution.

5.1 Introduction

The essential role of erythrocytes as oxygen carriers is historically well established, however, their function to aggregate with consequences on homeostasis is under debate. The aggregation property of red blood cells (RBC) is mainly considered to be pathophysiologic, since aggregation is elevated in many disease states such as diabetes mellitus[1] and hypertension[2].
Current understandings of blood rheology suggest complex mechanisms related to red blood cell *hyper*-aggregation. RBC *hyper*-aggregation is the main cause of increased blood viscosity under low shear conditions[3]. Increased aggregation is expected to augment the energy cost for breakdown of aggregates as blood approaches the microcirculation[4]. Enhanced RBC aggregation tends to promote axial accumulation of RBC in blood vessels, resulting in a less–viscous, plasma–rich region near vessel walls[5]. Decreased local viscosity of the marginal layers in blood vessels might be associated with decreased pressure gradients and hence lower wall–shear stresses for some vessels, thereby affecting vascular control mechanisms that are modulated by shear stress. Studies investigating the response of flow adapted endothelial cells, either in vivo or in vitro, demonstrated that positive or negative variation in shear stress at the vascular wall leads within minutes to membrane depolarization, increased intracellular Ca^{2+}, nitric oxide and reactive oxygen species generation[6]. In addition to synthesis and release on demand, several stored compounds are secreted during mechanical endothelial cell stimulation, in a Ca^{2+} dependent way. Elevation in intracellular Ca^{2+} triggers release of several vasoactive factors and factors involved in hemostasis and thrombolysis: nitric oxide (NO), prostacyclins, von Willebrand factor, tissue factor, tissue plasminogen activator, adhesion molecules and chemoattractant proteins[7]. In this respect, increased RBC aggregation was reported to result in diminished nitric oxide–dependent vascular control and decreased endothelial NO synthase expression[8].

To date and rather remarkable, the scientific approach to unravel this issue has completely ignored the pathogenic potential of low erythrocyte aggregation states. Some authors have suggested that normal levels of aggregation may serve homeostasis, having functional significance for normal physiology, as red cell aggregation is normally present in humans and other "athletic" species[9,10]. This hypothesis, however, has never been investigated before, and also, never been placed in a clinical relevant context.

The pathogenicity of low erythrocyte aggregation could have major implications for hemodiluted patients. This situation routinely occurs in cardiac patients undergoing on–pump cardiopulmonary bypass who are severely hemodiluted due to therapeutic preoperative isovolemic hemodilution, priming of the extracorporeal circuit and large fluid infusions peri–operatively. Excessive hemodilution prevails also during sustained fluid resuscitation in traumatic–hemorrhagic shock patients. In addition to the con-

sequences of hypoxic stress, the implications of low erythrocyte aggregation during acute hemodilution might prove to be essential for a full understanding of microcirculation impairment and deteriorated tissue perfusion in these patients. Considering the sensitivity of the vascular endothelium to variations in blood rheology, we hypothesized that low erythrocyte aggregation will be responsible for activating vascular endothelium during acute isovolemic hemodilution.
In this study we addresses the pathophysiology of acute isovolemic hemodilution in a clinical relevant animal model, studying hypoxic oxidative stress, red blood cell aggregation, and subsequent vascular endothelial activation.

5.2 Methods

This study was set up as a comparative, controlled, pseudo–double blind animal study, including a total of 15 adult pigs (60–80 kg). The experiments were in accordance with institutional and legislator regulations and approved by the local Committee for Animal Experiments. Two colloid solutions commonly used in clinical practice as plasma expanders were taken to induce acute isovolemic hemodilution (IHD). This experimental design was also based on our previous studies showing different effects of different molecular weight of hydroxyethyl starches (HES) on human red blood cells, with a pro–aggregatory effect increasing with the molecular weight of the colloid[11,12]. The animals were randomized in three groups:

- group 1 (n=6) 30 ml/kg isovolemic exchange transfusion with HAES–sterile 3% (HES 200/0.5, median molecular weight 200 kD, supplemented with Ringer's lactate to a final concentration of 3%).

- group 2 (n=6): 30 ml/kg isovolemic exchange transfusion with Voluven 3% (6% HES 130/0.4, median molecular weight 130 kD, supplemented with Ringer's lactate to a final concentration of 3%).

- group 3 (n=3): control group sham–operated animals.

Anaesthesia was induced with ketamine (i.m. 10 mg/kg) and diazepam (i.m. 1 mg/kg). Before intubation, the ventilation was performed using a mixture of O_2 and isoflurane 4%. After tracheal intubation, ventilation was performed with isoflurane 1.5–2%.
Isovolemic hemodilution was induced after cannulation of the jugular vein and carotid artery, by infusing HES at the arterial site, and a simultaneous withdrawal of an equal volume of blood. The drops in Hematocrit (Hct) and Hemoglobin (Hb) were monitored throughout the experiment, and adjusted to a constant value of 40% of the initial value (Fig. 5.1). No inotropic support was included in the protocol.
After 3 hours of maintaining the isovolemic hemodilution, tissue biopsies were obtained from the small intestine (ileum, luminal site), a randomly selected kidney

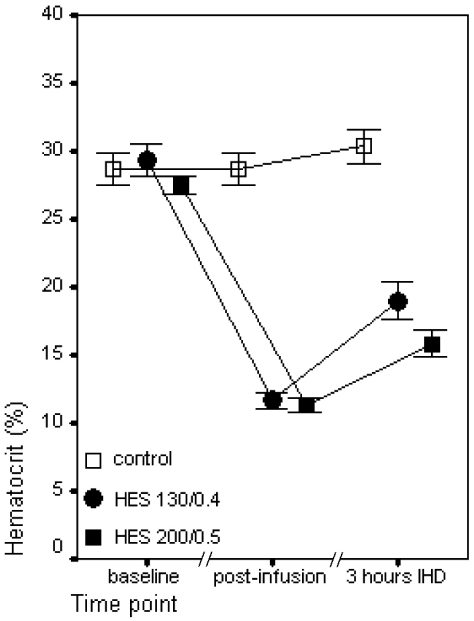

Figure 5.1: Hematocrit (%) variation during three hours of acute IHD, infused with either 3% HES 130/0.4 solution or 3% HES 200/0.5 solution. The controls are represented by sham–operated animals. The values are represented as mean (symbols) and standard error of the mean (bars).

(cortex), liver, lung, and heart. The biopsies were snap frozen in liquid nitrogen and stored at –80° C for real time RT–PCR measurements and histological assessments. Blood samples were collected at three time points: baseline (5 min after placement of the cannulae), post–infusion (5 min after induction of isovolemic hemodilution), and at the end of the experiment (3 hours of isovolemic hemodilution).

Test of red blood cell aggregation

The RBC aggregation measurements were performed on fresh arterial blood samples, using a Laser–assisted Optical Rotation Cell Analyzer (LORCA R&R Mechatronics, Hoorn, The Netherlands), and quantified as Aggregation Index (AI). This method closely mimics the in vivo blood flow conditions by applying a large range (0 to 500 s^{-1}) variations in shear rate and measuring the response in erythrocyte aggregation as indicated by the variation in the backscattered intensity from the blood layer [13]. In short, for the determination of red cell aggregation, the blood was brought under a shear rate of 500 s^{-1}, after which the shear was stopped. The backscattered intensity from the blood layer was measured during 120 s after shear stop. The intensity drops because of red blood cell aggregation [14].

Viscosity measurements of plasma samples were performed with an automated dy-

namic shear rheometer with cone–plate geometry (AR1000 Rheometer, TA Instruments). During measurements the temperature was set at 37° C and the shear rate of operation at $100\,s^{-1}$.

Test of hypoxic oxidative stress

Plasma Malondialdehyde (MDA) – enzymatic detection, according to the method described by Esterbauer and Cheeseman[15].

H_2O_2 production in bioptic tissues: a fluorophore–nitroxide (Molecular Probes, Eugene, OR, USA) was used to image ex–vivo superoxide and hydroxyl radicals generated by cells[16]. The reaction of fluorophore–nitroxide with superoxide results in a loss of electron spin resonance signal intensity concurrent with an increase in fluorescence emission. The fluorophore–nitroxide also reacts with methyl radicals generated by the reaction of hydroxyl radicals with DMSO[17].
Biopsies from tissue of approximately $2\,mm^3$ and dry weight of 2–5 mg were incubated for 10 minutes in a microtiterplate in 50 μl of 0.1 M Tris–HCl buffer (pH 8.0) containing 2.5 mM pyruvate and 5 mM succinate to stimulate mitochondrial activity[18]. Then 50 μl Tris–buffer containing 2 μM fluorescamine and DMSO (final concentration 2.5%) was added. The reaction was started after the addition of 5 μl FeII-EDTA (final Fe concentration 2 μM) in Tris buffer. In this way, both superoxide and hydroxyl radicals were converted and measured as H_2O_2[19]. The biopsies were incubated in this mixture for 10 min at room temperature on a plate shaker. After removal of the biopsies the fluorescence was measured in a multilabel counter (Victor2, EG&G Wallac, Turku, Finland) by using 390 nm excitation and 510 nm emission filters. Standard curves were obtained by adding known amounts of H_2O_2 to the assay medium.
During incubation hemoglobin was released from the biopsies, resulting in quenching of the fluorescence signal. Thus, a separate standard curve was prepared including stepwise diluted hemoglobin ranging from 0.1 to 1.2 g/L. The linear relationship between hemoglobin concentrations and fluorescence signal was used to correct for the hemoglobin signal quenching. Hemoglobin concentration in the supernatant of the incubated biopsies was measured by the method of Harboe[20]. Finally, measured H_2O_2 concentration was corrected for the dry weight of the biopsy.

Diaminobenzidine (DAB) staining – the production of H_2O_2 by cells in paraformaldehyde–fixed sections of ileum mucosa was histochemically demonstrated by incubating them for 30 min with 25 mg DAB/50 ml Tris/HCL pH 7.6, at 60° C. Catalase (150 μg/ml, 1400 U/ml) inhibited the reaction, indicating that H_2O_2 was required to produce the chromogenic DAB staining.

Endothelial activation: Real–Time Quantitative Taqman RT–PCR on von Willebrand factor, E–Selectin, P–Selectin, and endothelial nitric oxide synthase (eNOS)

gene expression in heart, lung, kidney, liver and intestinal tissue biopsies.
Total RNA was extracted using RNeasy Mini Kits (Qiagen, Venlo, The Netherlands), as recommended by the supplier. Total RNA was treated with 2 U of DNase I (RNase–Free DNase, Qiagen, Venlo, The Netherlands) in a volume of 15 μl to remove contaminating DNA (15 min at 37° C). First–strand cDNA synthesis: the mix of RNA (1 μg), 0.25 μg random hexamer primers and 2 ng of dNTPs (Promega, Leiden, The Netherlands) was heated for 5 min at 65° C and incubated on ice for at least 1 min, subsequently. The master mix [200 U SuperScript III (Invitrogen, Breda, The Netherlands) with 4 μl of 5× first–strand buffer, 1 μl 0.1 M dithiothreitol, and 40 U RNaseOUT ribonuclease inhibitor (Invitrogen)] was added to the samples in a total volume of 20 μl; finally a reverse transcriptase program was performed (5 min at 25° C, 60 min at 50° C, 15 min at 70° C, $\sim$ at 4° C).
Quantitative PCR amplifications were performed on an ABI Prism 7900HT Sequence Detection System (Applera Nederland, Nieuwekerk a/d IJssel, The Netherlands). Primers and probes for von Willebrand factor, E– and P–Selectin, eNOS, CD31 (endothelial marker) and GAPDH (house keeping gene) were developed commercially (Custom TaqMan® Assays, Applied Biosystems–Applera Nederland BV, Nieuwekerk a/d IJssel, The Netherlands). The mRNA coordinates for the exon–exon boundaries were determined by aligning the human genomic sequences with pig mRNA sequences (Spidey alignment program, `http://www.ncbi.nlm.nih.gov`). As a precaution to prevent amplification of genomic DNA, primer/probe sequences were chosen such that they span exon junctions or lie in distant exons separated by long introns. The PCR step contained 1 μl of the appropriate RT reaction, 10 μl of TaqMan universal PCR master mix (Applied Biosystems), 200 nM primers, and 100 nM TaqMan probe in a final volume of 20 μl. The PCR cycling conditions were 2 min at 50° C, 10 min at 95° C, and 40 two–step cycles of 15 s at 95° C and 60 s at 60° C. All samples were assayed in triplicate.
Relative quantification of the mRNA levels was done by subtracting the GAPDH C_T (threshold cycle) from the investigated gene C_T value ($\Delta C_T = C_{T\ \mathrm{gene}} - C_{T\ \mathrm{GAPDH}}$). Results were normalized with the average value of the respective gene in control sham–operated animals, arbitrarily set to 1. Results were finally expressed as $2^{-\Delta C_{T\ \mathrm{gene}}} / 2^{-\Delta C_{T\ \mathrm{CD31}}}$ which represents an index of the relative amount of mRNA expressed in each tissue, corrected for the number of endothelial cells presented in each biopsy.

Plasma concentrations of endothelial vWF were investigated by means of ELISA (Coamatic von Willebrand Factor kit, Nodia BV, Amsterdam, The Netherlands).

Statistical Analysis

The statistical analysis was performed using SPSS (Statistical Package for the Social Sciences). Before analysis, the data was tested for distribution according to

Kolmogorov–Smirnov goodness of fit test. The variations over the study period were investigated using repeated measures ANOVA. To investigate differences between groups, continuous variables where compared by means of parametric (Student T Test) or nonparametric tests (Mann–Whitney). Correlation between non–parametric variables was performed with Sperman's correlation test. Results are presented as mean±SEM (unless stated otherwise). Statistical significance was accepted at $p<0.05$.

5.3 Results

Immediately post–infusion the hematocrit (Hct, Fig. 5.1) reached 39.9±1.9% of baseline values in HES 130/0.4 hemodiluted animals and 41.3±2.2% of baseline values in HES 200/0.5 hemodiluted animals, but recovered by the end of the 3 experimental hours to 65.5±5.9% and 57.9±4.3% of baseline values, respectively. The extraordinary compensating capacity of the circulating number of erythrocytes was probably achieved by way of mobilizing spleen–trapped erythrocytes. This observation was supported by a smaller size and pale color of spleens in hemodiluted animals, as compared with those of sham–operated animals. To exclude the possibility of hemoconcentration due to loss of infused fluid through urine or extravascular extravasations, we performed plasma viscosity measurements. The baseline plasma viscosity levels (1.7±0.05 mPa.s) dropped in the hemodiluted animals immediately after infusion (1.39±0.06 mPa.s HES 130/0.4; 1.4±0.07 mPa.s HES 200/0.5) and remained low until the end of the experiment (1.35±0.13 mPa.s and 1.44±0.06 mPa.s, respectively) proving a comparable level of plasma dilution during the entire experiment.

	Baseline	Post–infusion	3 hours IHD
MAP (mmHg)			
controls	71.8±13.1	64.3±6.3	53.5±2.7
HES 130/0.4	71.0±16.6	46.1±4.8**	48.3±5.5
HES 200/0.5	67.3±9.0	52.6±17.4	49.5±5.5
HR (beats/min)			
controls	100±4	99±1	106±9
HES 130/0.4	90±10	108±13	138±11**
HES 200/0.5	91±9	106±10	124±22
Arterial PO_2 (mmHg)			
controls	70.1±2.8	70.7±3.1	69.1±1.6
HES 130/0.4	63.9±6.5	70.1±6.8	70.4±5.3
HES 200/0.5	62.4±7.1	68.4±7.8	69.6±7.1

Table 5.1: Mean arterial pressure (MAP), heart rate (HR) and arterial partial oxygen pressure (PO_2) during three experimental hours of isovolemic hemodilution

Hemodynamics

Mean arterial pressure (MAP) and heart rate (HR) were monitored throughout the experiment (Table 5.1). MAP decreased gradually and significantly (Wilks Sig.<0.001) in all animals during the experiment. Immediately post–infusion, MAP was significantly lower in the HES 130/0.4 group (Mann–Whitney p=0.024) than the control group; after 3 hours of hemodilution no significant differences were seen anymore. The heart rate increased gradually, with a stronger rise registered in hemodiluted animals (Wilks Sig. p=0.009). At the end of experiment, the HES 130/0.4 group had significantly higher heart rates than the control group (Mann–Whitney p=0.024).

RBC Aggregation

RBC Aggregation (Fig. 5.2) decreased significantly after induction of hemodilution (Wilks Sig.=0.002), with an overall lower aggregation index (AI) in the experimental animals as compared with sham–operated animals (between subjects effect sig.=0.001). In HES 130/0.4 group, AI dropped to 39.2±4.8% of baseline values and maintained low during the experiment with values of 37.05±3.3% of baseline values at the end of experiment. In HES 200/0.5, AI declined post–infusion to 49.3±5.9% of baseline values and maintained low with 47.7±6.5% of baseline values at the end of experiment. Although the AI tended to be higher in the HES 200/0.5 group than in the HES 130/0.4 group, the differences were not significant at any time point.

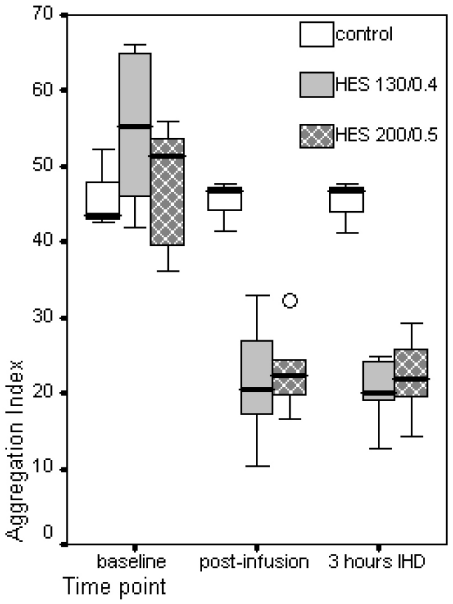

Figure 5.2: RBC aggregation during three hours of acute IHD, infused with either 3% HES 130/0.4 solution or 3% HES 200/0.5 solution. The controls are represented by sham–operated animals. The boundary of the box closest to zero indicates the 25^{th} percentile, the line within the box marks the median of 6 measurements, and the boundary of the box farthest from zero indicates the 75^{th} percentile. Whiskers above and below the box indicate the 90^{th} and 10^{th} percentiles. Symbol ∘ represents the outliers.

Hypoxic oxidative stress

Arterial PO_2 increased moderately but not significantly after hemodilution (Table 5.1), expressing either an improvement in pulmonary gas exchange or a decreased diffusional oxygen exit.

Plasma Malondialdehyde (MDA), (Fig. 5.3a) dropped significantly right after infusion, due to the dilution effect. The relative increase in plasma MDA during the 3 experimental hours was comparable in the sham–operated animals ($0.34\pm0.09\,\mu$mol), HES 130/0.4 infused animals ($0.30\pm0.16\,\mu$mol) and HES 200/0.5 infused animals ($0.40\pm0.18\,\mu$mol).

Oxygen radicals production (Fig. 5.3b). All animals showed a significantly higher H_2O_2 production in abdominal organs (ileum, kidney, liver) than in heart and lung tissues. Oxygen radicals production was comparable in all animals with no significant difference at any time point between hemodiluted and sham–operated animals.

A DAB staining of H_2O_2 producing cells in the ileum was performed, as the ileum seemed to be one of the organs exposed to oxidative stress. Fig. 5.3(c,d) shows a similar villi morphology, comparable staining intensity and distribution of H_2O_2 producing cells in both hemodiluted (Fig. 5.3c) and sham–operated animals (Fig. 5.3d).

Vascular endothelial activation

Von Willebrand Factor (vWF) mRNA (Fig. 5.4a) was significantly up–regulated in HES 130/0.4 hemodiluted animals when compared with sham–operated animals in all organs studied (Mann–Whitney: ileum, kidney, lung and heart p=0.024, liver p=0.048). The same outcome was found in HES 200/0.5, with the exception of the lungs, where differences did not reach significance (Mann–Whitney: ileum, kidney, and heart p=0.024, liver p=0.048, lung p=0.095). vWF mRNA responses did not differ between HES 130/0.4 and HES 200/0.5 treated animals.

Plasma vWF systemic release (Fig. 5.4b) translates the information found at mRNA level. Indeed, the relative increase in vWF plasma concentrations during three experimental hours in HES 130/0.4 group ($30.32\pm4.6\%$) and in HES 200/0.5 group ($27.9\pm1.3\%$) were significantly higher than the control values ($0.1\pm0.01\%$) in sham–operated animals (Mann–Whitney p=0.024 for both comparisons).

E–Selectin mRNA (Fig. 5.5a) was significantly up–regulated in the ileum and lungs of HES 130/0.4 hemodiluted animals, as compared with the sham–operated ones (Mann–Whitney p=0.048, and p=0.024, respectively). HES 200/0.5 hemodilution resulted in significantly up–regulated E–Selectin mRNA in the ileum (Mann–Whitney p=0.024)

when compared with control levels.

Sperman's correlation		Aggregation Index post–infusion
P Selectin–ileum	Correlation Coefficient	–0.614
	Sig. (2–tailed)	0.015
vWF–ileum	Correlation Coefficient	–0.539
	Sig. (2–tailed)	0.038
eNOS–ileum	Correlation Coefficient	–0.486
	Sig. (2–tailed)	0.066
vWF–liver	Correlation Coefficient	–0.546
	Sig. (2–tailed)	0.035
P Selectin–lung	Correlation Coefficient	–0.582
	Sig. (2–tailed)	0.023
P Selectin–heart	Correlation Coefficient	–0.496
	Sig. (2–tailed)	0.060
vWF–heart	Correlation Coefficient	–0.632
	Sig. (2–tailed)	0.011

Table 5.2: Statistical significant correlations found between RBC aggregation and markers of endothelial activation in different organs

P–Selectin mRNA (Fig. 5.5b) was up–regulated significantly in the ileum and lungs of both groups of hemodiluted animals (HES 130/0.4: ileum, lung Mann–Whitney p=0.024; HES 200/0.5: Mann–Whitney ileum p=0.024, lung p=0.048). Additionally for HES 200/0.5, the levels measured in the kidney reached significance when compared with controls (Mann–Whitney p=0.048).

Endothelial nitric oxide synthase (eNOS) mRNA (Fig. 5.6) was up–regulated significantly in the ileum and lungs in the HES 130/0.4 group (Mann–Whitney p=0.024 for both organs). In the HES 200/0.5 group, eNOS was up–regulated significantly only in the lungs (Mann–Whitney p=0.024).

Correlations

A significant negative correlation was found between the RBC aggregation index and levels of different markers of endothelial activation (Table 5.2).

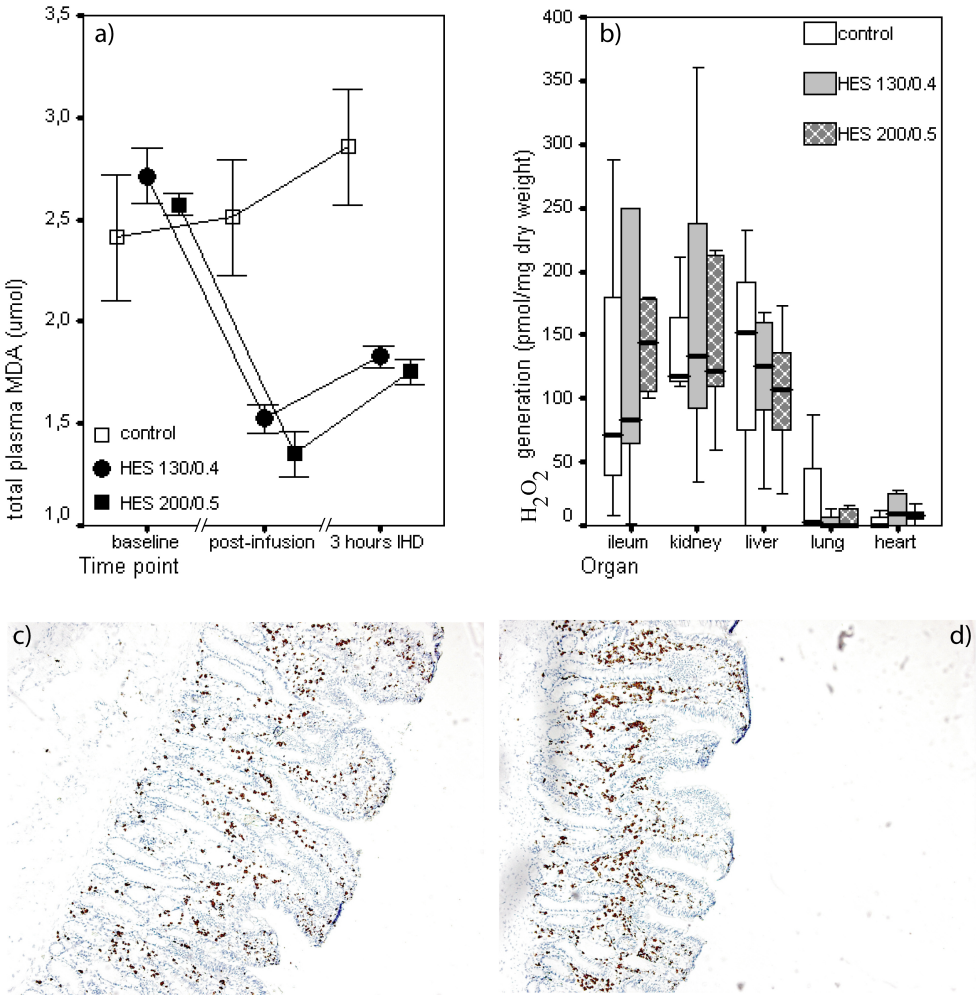

Figure 5.3: Hypoxic oxidative stress during 3 hours of acute IHD **(a)** *Plasma Malondialdehyde (MDA): The values are represented as mean (symbols) and standard error of the mean (bars);* **(b)** *Hydrogen peroxide (H_2O_2) production in heart, lung, kidney, liver, ileum tissue biopsies. Box plots graph data represent statistical values (see legend Fig. 5.2).* **(c)** *Diaminobenzidine (DAB) staining of H_2O_2–producing cells (brown coloration) in paraformaldehyde–fixed sections of ileum mucosa of hemodiluted and* **(d)** *sham–operated animals.*

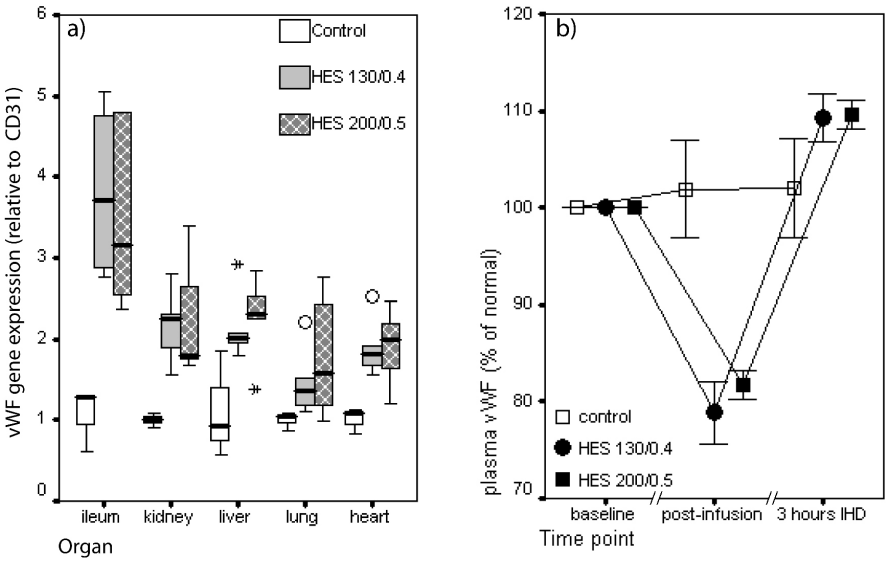

Figure 5.4: von Willebrand factor (vWF) **(a)** *vWF relative gene expression of in the heart, lung, kidney, liver, ileum tissue biopsies after 3 h IHD. HES 130/0.4 Mann-Whitney: ileum, kidney, lung and heart p=0.024, liver p=0.048. HES 200/0.5 Mann–Whitney: ileum, kidney, and heart p=0.024, liver p=0.048, lung p=0.095.* **(b)** *vWF plasma concentrations.*

5.4 Discussion

Using our experimental model of acute isovolemic hemodilution we documented an immediate pro–thrombotic and pro–inflammatory endothelial activation in heart, lung, kidney, liver, and ileum, accompanied by a dramatic drop in erythrocyte aggregation. Erythrocyte aggregability correlated significantly with markers of endothelial activation suggesting a causality effect.

The dynamic rheological properties of blood are defined mainly by the coordinated self–organization of RBCs advancing in the arterio–venular direction[21]. RBC *hyper*-aggregation is nowadays a generally recognized pathogenic factor, mainly due to clinical observation of increased RBC aggregation during disorders associated with macro and/or microvascular impairment, e.g. hypertension, diabetes mellitus, and chronic venous insufficiency[1,2,22]. *Hypo*-aggregation of RBCs has been never described in a pathologic context. Given the strong conditioning effect of RBC aggregation on blood rheology, and thus on mechanic endothelial activation, we hypothesized low RBC ag-

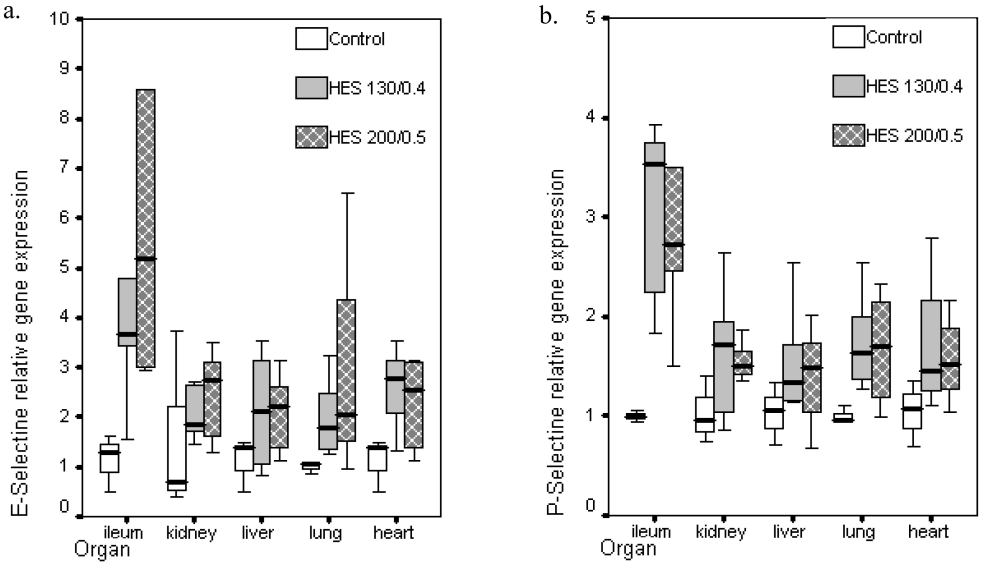

Figure 5.5: E–Selectin **(a)** *and P–Selectin* **(b)** *relative gene expression of in the heart, lung, kidney, liver, ileum tissue biopsies after 3 h IHD. E–Selectin: Mann–Whitney HES 130/0.4: ileum p=0.048, lungs p=0.024; HES 200/0.5: ileum p=0.024. P–Selectin: Mann–Whitney HES 130/0.4: ileum, lung p=0.024; HES 200/0.5: ileum p=0.024, lung p=0.048, kidney p=0.048.*

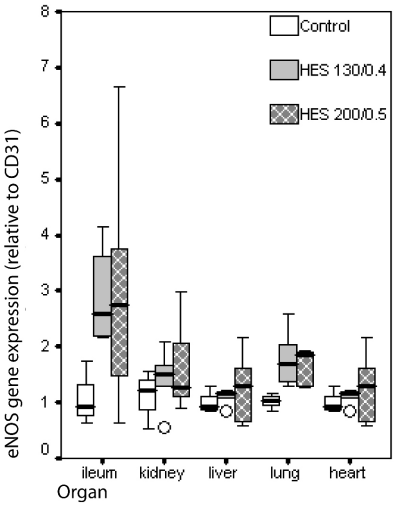

Figure 5.6: Endothelial nitric oxide synthase (eNOS) relative gene expression in the heart, lung, kidney, liver, ileum tissue biopsies after 3 h IHD. HES 130/0.4 Mann–Whitney: ileum, lungs p=0.024. HES 200/0.5 Mann–Whitney lungs p=0.024.

gregation to be a pathogenic co–factor in endothelial activation during acute isovolemic hemodilution. To verify this hypothesis, we induced acute isovolemic hemodilution in an "aggregating species", the pig[23], and investigated simultaneously the hypoxic oxidative stress, red blood cell aggregation, and gene regulation of von Willebrand factor, E– and P–Selectin, and eNOS, as markers of endothelial activation.
Hemodilution, by reducing the number of circulating RBCs is expected to decrease the oxygen–carrying capacity of blood and oxygen delivery to the tissue. However, during moderate levels of hemodilution, reduction of the systemic hematocrit up to 50% is compensated with an increased blood flow velocity and decreased diffusional oxygen exit from arterioles, resulting in augmented or maintained oxygen delivery to tissue[24]. In addition, reduction of systemic Hct during intentional hemodilution is not mirrored at the microcirculatory level, with capillary Hct sustained near control levels[25], thus maintaining tissue oxygenation.
In an experimental animal study, Deem en al.[26] showed that acute normovolemic hemodilution in healthy rabbits resulted in improved gas–exchange efficiency, as shown by higher arterial PO_2, lower alveolar–arterial PO_2 difference, and increased expired NO. They postulated that the improvement in oxygenation appeared to be related to increased uniformity of pulmonary blood flow, and/or an increase in concentration of the vaso– and bronchodilator substance NO. Our data support this assumption and consistently show an up–regulation of eNOS in the lung tissue during acute hemodilution.
In our approach to detect changes in tissue oxygenation, we tested ex–vivo the mitochondrial (dys)function in the vital organs (heart, lung, kidney, liver, ileum) of hemodiluted animals, reflected by the production of reactive oxygen species when oxidizing pyruvate and succinate. Thus, we aimed at detecting mitochondria that were pre–exposed to hypoxia during hemodilution. The production of superoxide and hydroxyl radicals, measured as H_2O_2 generation, was similar at all time points in sham–operated and hemodiluted animals, which indicates that a similar hypoxic oxidative stress was present, and oxygen delivery to the tissue during hemodilution was maintained. However, different organs seemed to have different exposure to hypoxia, with a more profound mitochondrial dysfunction in abdominal organs (ileum, kidney, liver) versus a preserved function of mitochondria in the myocardium and lung tissue. The results found in tissue biopsies were mirrored by the plasma MDA determinations, that showed similar relative increase in systemic lipid peroxidation products during three experimental hours when hemodiluted animals and sham–operated animals were compared. These results suggest that, at least in this animal model, the perioperative stress and the anesthetic management are more important triggers of oxidative stress in abdominal organs, than hemodilution per–se.
Because hypoxic stress seems to be negligible in this model of acute isovolemic hemodilution, we suggest that the effects observed in endothelial activation were mainly due to the drop in RBC aggregation.

Erythrocyte aggregation and endothelium–dependent pro–thrombotic activation

First reliable observations on the involvement of red blood cell in the process of clot formation were made by Turitto et al.[27] who showed that under flow conditions platelet adhesion and thrombus formation increase as hematocrit values increase from 10% to 70%. They hypothesized that red cells may have a significant influence on hemostasis and thrombosis and the nature of this effect is apparently related to the flow conditions. More recently, it was demonstrated that erythrocytes markedly increase platelet eicosanoid formation, promote release of intracellular platelet granule components, and induce recruitment of additional platelets from the microenvironment into the forming thrombus[28,29].
The data presented in this study suggest a new pathway for erythrocyte involvement in clot formation: due to their function to aggregate, erythrocyte could modulate endothelial activation with von Willebrand factor release, with a subsequent pro–thrombotic effect. von Willebrand factor, which is stored in the endothelial Weibel–Palade storage granules, has unique biomechanical properties and a critical biological role as an adhesive protein. It mediates the adhesion of platelets to an injured vascular wall by binding on platelet surface and to collagen in the subendothelium. vWF is one of the most potent activators of platelets. Activation of platelets causes them to release additional vWF from their α–storage granules. Increased levels of plasma von Willebrand factor contribute directly to thrombosis, impeding the normal flow of circulating blood[30]. In our experiment, acute isovolemic hemodilution was followed by a dramatic drop in red blood cell aggregation, which resulted in immediate pro–thrombotic endothelial activation as shown by a systemic increase in plasma vWF levels. Analysis of vWF mRNA expression levels in different vital organs showed a concomitant up–regulation in heart, lungs, kidney, liver, and small intestine. In addition, low red blood cell aggregation states were significantly associated with high vWF mRNA expression in heart, liver and ileum suggesting maybe a causality effect. An understanding of how disturbed blood flow might lead to disease is now emerging. Transferring this knowledge to a clinical relevant situation, as the one of the patients undergoing on–pump cardiopulmonary bypass, we hypothesize that lower incidence of thrombotic events could be achieved by avoiding excessive peri–operative hemodilution.

Low erythrocyte aggregation and endothelium–dependent pro–inflammatory response

The presence of a high RBC aggregation proved already its relevance in diagnosing the patients' inflammatory status, using clinical observations of positive correlations between enhanced RBC aggregation and high plasma levels of C–reactive protein and fibrinogen[31]. There is also evidence that RBC *hyper*-aggregation enhances the ten-

dencies of leukocytes to adhere to the postcapillary endothelium, a process recognized as essential in inflammation. Pearson et al.[32] reported that increased RBC aggregation was associated with increased adhesion of white blood cells to the endothelium, possibly because of an enhanced probability of contact between leukocytes and the postcapillary venular wall.
In this study we discovered that RBC *hypo*-aggregation, documented in our model of acute isovolemic hemodilution, was statistically significant correlated with up–regulation of endothelial adhesion molecules, E– and P–Selectins, especially in lungs and small intestine. E– and P–Selectins belong to the Selectin family of adhesion molecules and both have been reported to increase in circulation or at lesion sites of several diseases reflecting endothelial activation. Selectins play important roles in the inflammatory responses by facilitating leukocyte rolling and leukocytes activation[33,34].
Translation of these data in clinical terms suggests that acute hemodilution may lead to inflammatory stress of pulmonary capillaries. Subsequent diffusion limitation may be expected. Similar, an increased inflammatory response in the small intestine associated with acute hemodilution, might contribute to a loss in barrier function of the intestinal mucosa with subsequent translocation of endotoxins and/or bacteria.

Conclusions

The data presented in this study show that acute isovolemic hemodilution definitely triggers endothelial activation. Since the effects of hypoxic oxidative stress seem to be negligible in this model, red blood cell *hypo*-aggregation could be considered as a new pathophysiologic mechanism which could be held responsible for pro–inflammatory and pro–coagulatory endothelial activation. We hypothesize that a reduced inflammatory response and a lower incidence of thrombotic events will be achieved by avoiding excessive peri–operative hemodilution during on–pump cardiopulmonary bypass.

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References

1. Martinez M, Vaya A, Server R, Gilsanz A, Aznar J. Alterations in erythrocyte aggregability in diabetics: the influence of plasmatic fibrinogen and phospholipids of the red blood cell membrane. *Clin. Hemorheol. Microcirc.* 1998;18:253–8.

2. Cicco G, Pirrelli A. Red blood cell (RBC) deformability, RBC aggregability and tissue oxygenation in hypertension. *Clin. Hemorheol. Microcirc.* 1999;21:169–77.

3. Baskurt OK and Meiselman HJ. Cellular determinants of low shear blood viscosity. *Biorheology.* 1997;34:235–247.

4. Vicaut E. Opposite effects of red blood cell aggregation on resistance to blood flow. *J. Cardiovasc. Surg. (Torino).* 1995;36:361–368.

5. Cokelet GR and Goldsmith HL. Decreased hydrodynamic resistance in the two-phase flow of blood through small vertical tubes at low flow rates. *Circ. Res.* 1991;68:1–17.

6. Fisher AB, Chien S, Barakat AI, Nerem RM. Endothelial cellular response to altered shear stress. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2001;281:L529–33.

7. Nilius B, Droogmans G. Ion Channels and their functional role in vascular endothelium. *Physiological Reviews.* 2001;4:1415–9.

8. Baskurt OK, Yalcin O, Ozdem S, Armstrong JK, and Meiselman HJ. Modulation of endothelial nitric oxide synthase expression by red blood cell aggregation. *Am. J. Physiol. Heart Circ. Physiol.* 2004;286:H222–H228.

9. Popel AS, Johnson PC, Kameneva MV, Wild MA. Capacity for red blood cell aggregation is higher in athletic mammalian species than in sedentary species. *J. Appl. Physiol.* 1994;77:1790–4.

10. Bishop JJ, Nance PR, Popel AS, Intaglietta M, Johnson PC. Effect of erythrocyte aggregation on velocity profiles in venules. *Am. J. Physiol. Heart Circ. Physiol.* 2001;280:222–36.

11. Morariu AM, Vd Plaats A, V Oeveren W, 'T Hart NA, Leuvenink HG, Graaff R, Ploeg RJ, Rakhorst G. Hyperaggregating effect of hydroxyethyl starch components and University of Wisconsin solution on human red blood cells: a risk of impaired graft perfusion in organ procurement? *Transplantation.* 2003;76:37–43.

12. Morariu AM, Gu YJ, Huet RC, Siemons WA, Rakhorst G, Oeveren WV. Red blood cell aggregation during cardiopulmonary bypass: a pathogenic cofactor in endothelial cell activation? *Eur. J. Cardiothorac. Surg.* 2004;26:939–46.

13. Hardeman MR, Goedhart PT, Lettinga KP. Laser-assisted optical rotational cell analyser (L.O.R.C.A.); I. A new instrument for measurement of various structural hemorheological parameters. *Clinical Hemorheology.* 1994;14:605–618.

14. Graaff R, Gu YJ, Boonstra PW, van Oeveren W, Rakhorst G. Analysis of red blood cell aggregation in cardio-pulmonary bypass (CPB) surgery. *Int. J. Artif. Organs.* 2004;27:488–94.

15. Esterbauer H, Cheeseman KH Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol.* 1990;186:407–21.

16. Pou S., Huang Y. I., Bhan A., Bhadti V. S., Hosmane R. S., Wu S. Y., Cao G. L. and Rosen G. M. A Fluorophore-Containing Nitroxide as a Probe to Detect Superoxide and Hydroxyl Radical Generated by Stimulated Neutrophils. *Anal. Biochem.* 1993;212:85–90.

17. Li B, Gutierrez PL, Blough NV. Trace determination of hydroxyl radical in biological systems. *Anal. Chem.* 1997;69:4295–302.

18. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL. Production of reactive oxygen species by mitochondria: central role of complex III. *J. Biol. Chem.* 2003;278:36027–31.

19. Li B, Gutierrez PL, Blough NV. Trace determination of hydroxyl radical using fluorescence detection. *Methods Immunol.* 1999;300:202–216.

20. Harboe A. Method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry. *Scand. Clin. Lab. Invest.* 1959;11:66–70.

21. Mchedlishvili G. Disturbed blood flow structuring as critical factor of hemorheological disorders in microcirculation. *Clin. Hemorheol. Microcirc.* 1998;19:315–25.

22. Krieger E, van Der Loo B, Amann-Vesti BR, Rousson V, Koppensteiner R. C-reactive protein and red cell aggregation correlate with late venous function after acute deep venous thrombosis. *J. Vasc. Surg.* 2004;40:644–9.

23. Windberger U, Bartholovitsch A, Plasenzotti R, Korak KJ, Heinze G. Whole blood viscosity, plasma viscosity and erythrocyte aggregation in nine mammalian species: reference values and comparison of data. *Exp. Physiol.* 2003;88:431-40.

24. Tsai AG, Friesenecker B, McCarthy M, Sakai H, Intaglietta M. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. *Am. J. Physiol.* 1998;275:H2170–80.

25. Lipowsky HH, and Firrell JC. Microvascular hemodynamics during systemic hemodilution and hemoconcentration. *Am. J. Physiol. Heart Circ. Physiol.* 1986;250:H908–H922.

26. Deem S, Hedges RG, McKinney S, Polissar NL, Alberts MK, Swenson ER. Mechanisms of improvement in pulmonary gas exchange during isovolemic hemodilution. *J. Appl. Physiol.* 1999;87:132–41.

27. Turitto VT, Weiss HJ. Red blood cells: their dual role in thrombus formation. *Science.* 1980;207:541–543.

28. Valles J, Santos MT, Aznar J, Marcus AJ, Martinez-Sales V, Portoles M, Broekman MJ, Safier LB. Erythrocytes metabolically enhance collagen-induced platelet responsiveness via increased thromboxane production, ADP release, and recruitment. *Blood.* 1991;78:154–162.

29. Santos MT, Valles J, Aznar J, Marcus AJ, Broekman MJ, Safier LB. Prothrombotic effects of erythrocytes on platelet reactivity: reduction by aspirin. *Circulation.* 1997;95:63–68.

30. Ruggeri ZM. Role of von Willebrand factor in platelet thrombus formation. *Ann. Med.* 2000;32:2–9.

31. Ami RB, Barshtein G, Zeltser D, Goldberg Y, Shapira I, Roth A, Keren G, Miller H, Prochorov V, Eldor A, Berliner S, Yedgar S. Parameters of red blood cell aggregation as correlates of the inflammatory state. *Am. J. Physiol. Heart Circ. Physiol.* 2001;280:H1982–8.

32. Pearson MJ, Lipowsky HH. Influence of erythrocyte aggregation on leukocyte margination in postcapillary venules of rat mesentery. *Am. J. Physiol. Heart Circ. Physiol.* 2000;279:H1460–71.

33. Ley K, Allietta M, Bullard DC, Morgan S. Importance of E-selectin for firm leukocyte adhesion in vivo. *Circ. Res.* 1998;83:287–94.

34. Kunkel EJ, Chomas JE, Ley K. Role of primary and secondary capture for leukocyte accumulation in vivo. *Circ. Res.* 1998;82:30–8.

Chapter 6

Organ Perfusion in Donation and Transplantation: Organ Viability in Brain Dead Donors.

Impact of Brain Death on Donor Kidneys: Early Progression of Endothelial Activation, Oxidative Stress and Tubular Injury

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Submitted to *Transplantation*

Abstract

Cerebral injury leading to brain death (BD) causes major hemodynamic instabilities in potential organ donors that may induce endothelial activation and affect post-transplant graft function. We investigated pro–coagulatory and pro–inflammatory effects of endothelial activation after BD with the subsequent oxidative stress and renal tubular injury.

Brain death was induced by slowly inflating over a period of 30 minutes of a balloon–catheter inserted in the extradural space. To assess time–dependant changes due to BD, rats (n=30) were sacrificed 0.5, 1, 2, or 4 hours after BD–induction and compared to sham–operated controls. E– and P–Selectins, fibrinogen mRNA were abruptly and progressively up–regulated from 0.5 hours BD onwards; P–Selectin membrane–expression was increased. Plasma von Willebrand factor was significantly higher after 2 hours and 4 hours BD, reflecting sustained platelet adhesion to the vascular–wall. Oxidative stress in kidneys was detectable only late, being significantly increased in 2 hours, and 4 hours groups. Urine heart–fatty–acid–binding–protein and N–acetyl–glucosaminidase, used as new specific and more sensitive markers of proximal and distal tubular damage, were found to be significantly increased after 0.5 hours, and maximum at 4 hours.

This study demonstrates immediate pro–coagulatory and pro–inflammatory activation of vascular endothelium after BD in kidney donor rats, proportional with the duration of BD. Oxidative stress measurements pointed at ischemia/reperfusion injury during protracted periods of BD. BD–related donor kidney damage was diagnosed after half hour of BD.

6.1 Introduction

To date, the retrieval of kidneys in brain death donation is primarily dependent on the logistics concerning the donor operation and the timing of the donor retrieval team. Following previous work that clearly documented the detrimental effect of a prolonged state of brain death, the clinicians recognize more and more the need to retrieve organs as soon as possible, avoiding unnecessary prolongation of organ procurement, in order to maintain viability.
Due to cerebral injury with subsequent brain death and loss of integrated neurological function, the potential organ donor is exposed to major physiologic derangements[1–3]. To maintain function, an aggressive, labor–intensive donor management is required throughout the ICU stay.
Endothelial activation in brain dead donors has gained lately considerably attention in the discussion concerning the pathological effects of brain death on donor organ quality prior to retrieval. The vascular endothelial phenotype is known to change dramatically under various pathophysiologic conditions, expressing cell adhesion molecules, releasing cytokines and substrates that promote thrombosis and inflammation. Under hemodynamic and rheological unstable conditions endothelial cells have demonstrated their ability to sense variations in mechanical forces such as shear stress, that appear as a consequence of blood flow and viscosity alterations[4,5]. Studies investigating the response of flow–adapted endothelial cells have shown that variation in shear stress was followed within minutes by membrane depolarization, increased intracellular Ca^{2+}, release of nitric oxide, reactive oxygen species, von Willebrand factor, prostacyclins, tissue factor, tissue plasminogen activator, adhesion molecules and chemoattractant proteins[6,7]. Previous studies suggest that an immune activation with increased endothelial cell activation and immediate early gene expression occurs after brain death induction[8–10]. Moreover, the expression of endothelial adhesion molecules (intercellular adhesion molecule–1 and vascular cell adhesion molecule–1) and the influx of leukocytes in the kidney is shown to occur faster and be more profound when hemodynamic instability in the brain dead donor is not corrected[11]. Furthermore, the non–specific inflammatory response activated during brain death was shown to accelerate acute rejection of organs procured from brain dead donors[12].
As an original contribution, this study aims to analyze and document the time sequence for the most early progression post–BD induction of pro–inflammatory and pro–coagulatory endothelium activation, oxidative stress and organ viability in brain dead rat kidney donors. We hypothesized that activated endothelium in the brain dead donor will express and release both pro–inflammatory and pro–coagulatory factors into circulation that will mediate inflammation, platelet adhesion and possibly promote microthrombosis. In addition, we expect that due to activation of endothelium and hypoxic stress, the oxidative stress and brain death–related organ dysfunction will arise early after BD–induction.

6.2 Methods

Animals and Experimental protocols

The experiments were in accordance with institutional and legislator regulations and approved by the local Committee for Animal Experiments. A total of 30 rats (adult male Fisher 344 rats, 260–300 g, Harlan, Zeist, The Netherlands) were studied. To assess time–dependant changes due to brain death (BD), the animals (n=6 per group) were sacrificed after 0.5, 1, 2, or 4 hours after induction of BD. Controls (n=6) consisted of sham–operated rats using a trepanation, however without inserting the balloon catheter to cause cerebral injury. Sham–operated rats remained ventilated and under anesthesia for half hour. All rats were sacrificed after completion of the experiment.

Surgical procedures

Animals were anesthetized using oxygen/nitrous oxide/isoflurane 5%; isoflurane was reduced to 2% after anesthesia induction. Corporeal temperature was maintained at 37° C. After frontolateral trepanation lateral of the bregma, a balloon catheter (0,75 ml 4F EMB, Edward Lifesciences ref 120404F) was inserted and slowly inflated over a time period of 30 min with 0.5 ml water using a syringe pump. After approximately 27 min., the rats became apneic and were mechanically ventilated (12–15 mmHg relief, 1–5 mmHg positive end–expiratory pressure, Zoovent CWC600AP; Triump technical services Ltd, United Kingdom.) through a tracheostoma (47/min frequency, 40% inspiration phase). After BD induction, anesthesia was stopped and the rats were ventilated with 100% O_2 for 30 min; subsequently ventilation was switched to O_2/air. Ten minutes before retrieval of organs, the rats were ventilated with oxygen/nitrous oxide/isoflurane 0.5% to allow muscle relaxation and laparotomy. Brain death was confirmed by the absence of brain stem reflexes, the pupillary reflex, the corneal reflex and an apnoea test. The mean arterial pressure was continuously measured and recorded using an intra–arterial blood pressure sensor (Truwave, Edwards Lifesciences, Irvine, USA, recorder Labview 5.1; National instruments Co., Austin, USA). A MAP lower than 80 mmHg was corrected by colloid infusion (10% hydroxyethyl starch, HAES, 37° C). Kidneys were retrieved after a flush with saline through the abdominal aorta, snap frozen in liquid nitrogen and stored at –80° C.
To study the pro–inflammatory response as a result of the induction of brain death we investigated endothelial gene expression of E– and P–Selectin (real time RT–PCR), and membrane expression of P–Selectin (immunohistochemistry). The pro–coagulatory response during brain death was assessed by investigating circulating levels of von Willebrand factor (ELISA) and fibrinogen gene expression.

Immunohistochemistry

P–Selectin staining (Table 6.1) was performed on cryosection, acetone fixed slides. A semi–quantitative evaluation of the P–Selectin endothelial expression was performed in a double–blind fashion by two independent pathologists in parallel. The semi–quantitative scoring system used for grading P–Selectin endothelial staining had a scale of 0 to 3 arbitrary units: 0 (none), 1 (mild), 2 (moderate), 3 (intense).
In addition, a P–Selectin positive platelets counting was performed, considering each time 30 randomly chosen glomeruli per slide and reporting the results as average platelet number per glomeruli.

Antibody	Type	Dilution	Incubation time	Company
primary antibody	rabbit polyclonal antibody against rat P–Selectin	1:25	1 hour	BD Pharmigen
2^{nd} antibody	goat anti–rabbit immunoglobulin antiserum	1:100	30 min	Vector Laboratories BA–1000
3^{rd} antibody	peroxidase conjugated rabbit anti–goat polyclonal antibody	1:100	30 min	Vector Laboratories BA–1000

Table 6.1: Immunohistochemical staining of P–Selectin on cryostat section, acetone fixed slides of rat kidney tissue. Antibodies were diluted in PBS containing 1% bovine serum albumin; 1% normal rat serum was added to the secondary antibodies. The peroxidase activity was developed using 3–amino–9–ethylcarboxide (AEC)/H_2O_2.

Real time reverse transcriptase PCR for Aα and Bβ fibrinogen chains, E– and P–Selectin gene expression were assessed using amplification primers designed with Primer Express software (Applied Biosystems, Foster City, USA). The primers sequence and product sizes are included in Table 6.2. Amplification and detection were performed with an ABI Prism 7900–HT Sequence Detection System (Applied Biosystems, Foster City, USA) using emission from Sybr green. All assays were performed in triplicate. Gene expression was normalized with the mean of β–actin mRNA content and calculated relative to controls using the relative standard curve method. Results were finally expressed as $2^{-\Delta C_T}$ (C_T threshold cycle).
Plasma vWF concentrations were measured by ELISA (Coamatic von Willebrand Factor kit, Nodia BV, Amsterdam, The Netherlands).

Detection of oxygen radicals production
A fluorophore–nitroxide was used to image oxygen radicals generated during ex–vivo incubation of kidney biopsies[13–15]. Both superoxide and hydroxyl radicals were con-

Gene	Forward primer sequences	Reverse primer sequences	Size
Aα fibrinogen	5'-GCTCTGTCCTCAGGGTTGAATTA-3'	5'-GCCTACCCGGAAGTGGTACTC-3'	73 bp
Bβ fibrinogen	5'-CGGCGGCTGGTGGTATAA-3'	5'-CTGTAAAGGCCACCCCAGTAGTAT-3'	71 bp
E–Selectin	5'-GTCTGCGATGCTGCCTACTTG-3'	5'-CTGCCACAGAAAGTGCCACTAC-3'	73 bp
P–Selectin	5'-TCTCTGGGTCTTCGTGTTTCTTATCT-3'	5'-GTGTCCCCCTAGTACCATCTGAA-3'	71 bp

Table 6.2: Primers sequence and product sizes used for Real time reverse transcriptase PCR for Aα and Bβ fibrinogen chains, E– and P–Selectin gene expression.

verted and measured as H_2O_2 [16]. Fluorescence was measured with a multilabel counter (390 nm excitation and 510 nm emission filters, Victor2, EG&G Wallac, Turku, Finland). Standard curves were obtained by adding known amounts of H_2O_2 to the assay medium. A separate standard curve was prepared including stepwise diluted hemoglobin. The linear relationship between hemoglobin concentrations and fluorescence signal was used to correct for the hemoglobin signal quenching. Hemoglobin concentration in the supernatant of the incubated biopsies was measured by the method of Harboe[17]. H_2O_2 concentration were corrected for the dry weight of the biopsy.

Kidney injury biomarkers

Urine heart–type fatty acid binding protein (H–FABP) – ELISA (HyCult Biotechnology B.V., Uden, The Netherlands). The kit has a minimum detection limit of 0.4 ng/ml and a measurable concentration range of 0.4–25 ng/ml. Samples were diluted 10 times before measurement.
Urine N–acetyl–glucosaminidase (NAG) – modified enzyme assay according to Lockwood[18] at pH 4.5 and corrected for non–specific conversion (HaemoScan, Groningen, The Netherlands).
Both NAG and H–FABP urine concentrations were corrected for dilution using urine creatinine values.

Statistical Analysis

The statistical analysis was performed using SPSS (Statistical Package for the Social Sciences).
Before analysis, the data was tested for distribution according to Kolmogorov–Smirnov goodness of fit test. To investigate differences between groups, continuous variables were compared by means of parametric (Student T Test) or nonparametric tests (Mann–Whitney). A p value smaller than 0.05 was considered statistically significant. Results are presented as mean±SEM (unless stated otherwise).

6.3 Results

Hemodynamics.

The blood MAP measured prior to balloon inflation was 116±3 mmHg. After 10±1 minutes from inflation, the blood pressure decreased sharply and remained in a hypotensive state (59±2 mmHg) for another 11±1 minutes. At the end of the brain death induction the recording showed a sharp peak (142±5 mmHg). 10.31±0.90 minutes after the onset of brain death the blood pressure decreased to 46±1 mmHg. Subsequently, a peak in blood pressure occurred, followed by a plateau at levels above 100 mmHg (Fig. 6.1).
Basal heart rate was 356±8 beats/minute (bpm). During inflation of the balloon a slight increase in heart rate was observed. After the peak in MAP had occurred, a decline in heart rate to its basal level was observed.

Donor management.

During mechanical ventilation the MAP remained at levels above 80 mmHg. Five animals needed colloid infusion (3 ml/kg) to correct for hypotension. All five responded well with a return to basal levels within three minutes. Thirty minutes after BD induction, the apnea test was found positive for all animals, with no spontaneous respiration. Corneal and pupillary reflexes were absent as well.

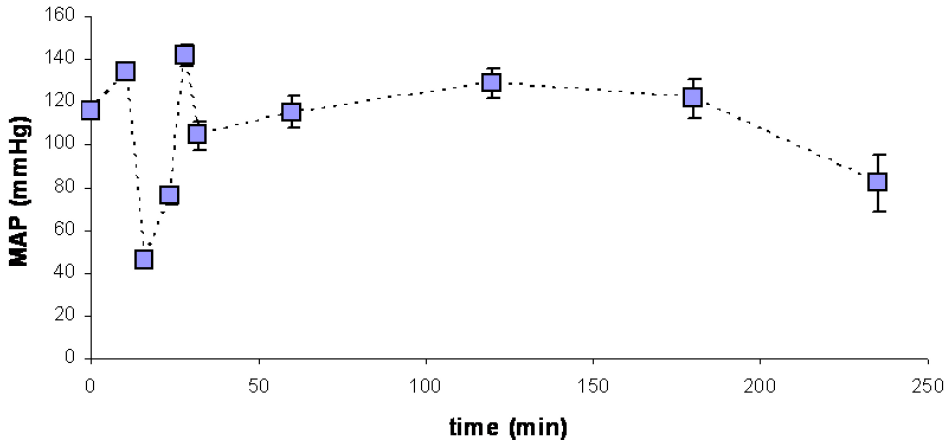

Figure 6.1: Mean arterial pressure (MAP) monitoring during and after progressive brain death induction. The values are represented as mean (symbols) and standard error of the mean (bars).

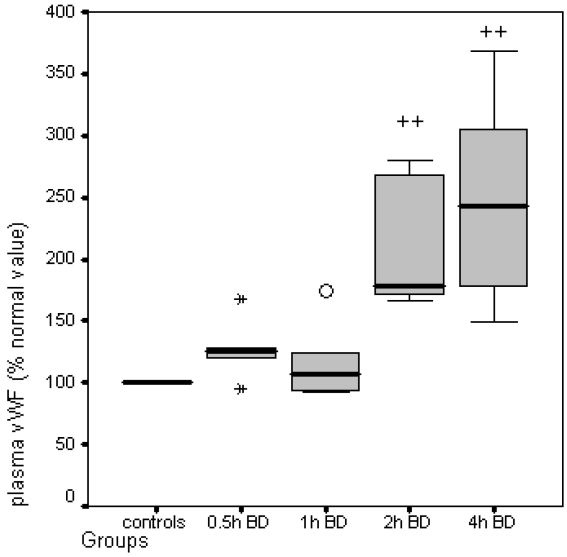

Figure 6.2: Concentrations of von Willebrand Factor (vWF) in plasma of the brain dead rats after 0.5, 1, 2, or 4 hours since brain death (BD) induction. The controls are represented by sham–operated animals. Box plots graph data represent statistical values. The boundary of the box closest to zero indicates the 25^{th} percentile, the line within the box marks the median of 6 measurements, and the boundary of the box farthest from zero indicates the 75^{th} percentile. Whiskers above and below the box indicate the 90^{th} and 10^{th} percentiles. Symbols ° and ⋆ represent the outliers and extremes, respectively. ++, + differences between BD group and controls group are significant at the 0.01 and 0.05 level, respectively.

Plasma von Willebrand factor (Fig. 6.2)
In the 0.5 h BD and 1 h BD groups, plasma vWF values remained in the normal range or increased moderately but only in sporadic cases. After 2 h of BD, however, vWF increased sharply, reaching significant different values in the 2 h BD (207±52%) and 4 h (248±88%) groups as compared with controls (Mann–Whitney p=0.002 for both time points).

Fibrinogen mRNA expression in rat kidney tissue (Fig. 6.3a,b)
Both Aα and Bβ fibrinogen chain mRNAs expression in renal tissue were highly and significantly up–regulated after 2 hours (Aα fibrinogen 22±12.9 fold induction, Mann–Whitney p=0.01; Bβ fibrinogen 7.8±4.6 fold induction, Mann–Whitney p=0.016) and 4 hours (Aα fibrinogen 86±36 fold induction, Mann–Whitney p=0.004; Bβ fibrinogen 54.6±29.6 fold induction, Mann–Whitney p=0.004) of brain death.

E–Selectin mRNA expression in rat kidney tissue (Fig. 6.4a) was up–regulated early, reaching already at 0.5 h post–BD folds ten times higher (10±3.5, Mann–Whitney p=0.004) than control values. The expression continued to increase in time, so

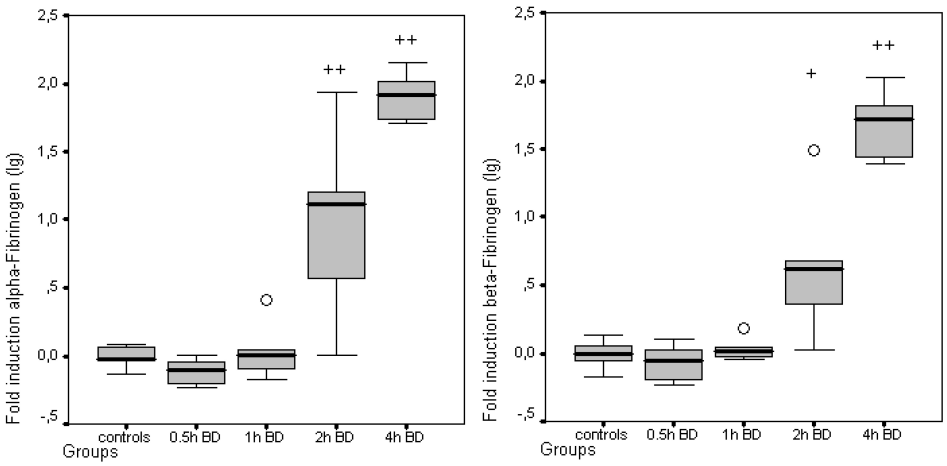

Figure 6.3: Relative gene expression (mRNA fold induction on a logarithmic scale) of Aα **(a)** *and Bβ* **(b)** *fibrinogen chains in the kidney tissue of brain-dead rats after 0.5, 1, 2, or 4 hours since brain death (BD) induction. The controls are represented by sham–operated animals. Box plots graph data represent statistical values (see Fig. 6.2).*

that the relative gene expression was 13.6±3.7 (fold induction) at 1 h of BD (Mann–Whitney p=0.002). After 2 h of BD the E–Selectin gene was strongly up–regulated (54.7±14.2 fold induction, Mann–Whitney p=0.002). The animals brain dead for 4 h had the most important up–regulation in E–Selectin gene expression (135.9±56.2 fold induction, Mann–Whitney p=0.002).

P–Selectin mRNA expression in rat kidney tissue (Fig. 6.4b) was significantly up–regulated starting with 1 h of BD (12.4±3.1 fold induction, Mann–Whitney p=0.002). P–Selectin gene expression increased progressively starting with 2 h BD (58.9±17.6 fold induction, Mann–Whitney p=0.002), with maximum values at 4 h BD (92.4±26.8 fold induction, Mann–Whitney p=0.002).

P–Selectin Immunohistochemistry (Fig. 6.5a,b)
P–Selectin endothelial membrane expression was absent in control samples (Fig. 6.5a), while in BD animal samples it started to be observed as early as half–hour after BD induction. The expression continued to increase, so that at 4 hours BD P–Selectin was omnipresent on the surface of vascular endothelial cells in the renal tissue (Fig. 6.5b). The arbitrary P–Selectin expression score at 4 h BD (2.2±0.2) was significantly higher (Mann–Whitney p=0.004) than the score in controls (0.33±0.22). Besides en-

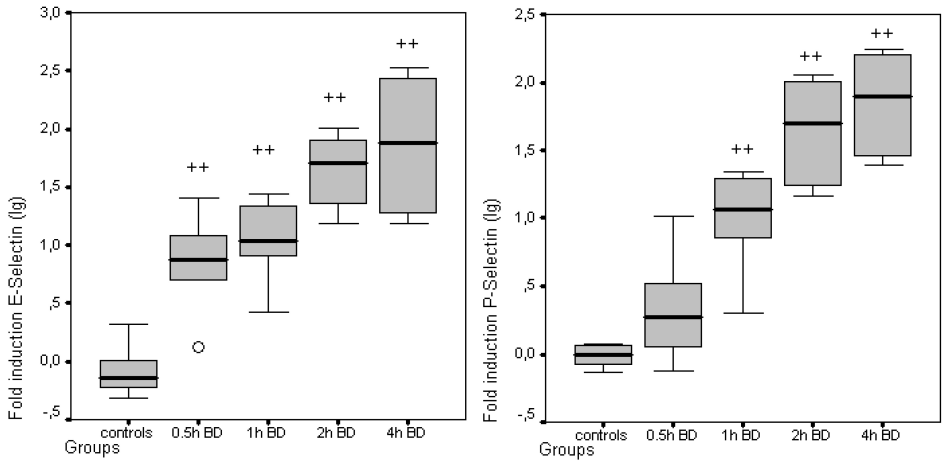

Figure 6.4: Relative gene expression (mRNA fold induction on a logarithmic scale) of E–Selectin **(a)** *and P–Selectin* **(b)** *in the kidney tissue of brain dead rats after 0.5, 1, 2, or 4 hours since brain death (BD) induction. The controls are represented by sham–operated animals. Box plots graph data represent statistical values (see Fig. 6.2).*

dothelial expression, P–Selectin was stained in platelets trapped in the glomeruli, after washing–out the organs. After 1 hour of BD the platelet count per glomeruli (3.09±0.6 platelets/glomeruli) was significantly higher (Mann–Whitney p=0.04) that the number of platelets trapped in the controls samples (0.96±0.6 platelets/ glomeruli).

Oxidative stress (Fig. 6.6)
H_2O_2 production in the kidney tissue increased non-significantly during the first hour of brain death (0.5 h BD 18.6±9.6; 1 h BD 51.6±21.4). H_2O_2 values became significantly higher than control values after 2 h BD (115.7±32, Mann–Whitney p=0.004). After 4 h BD, H_2O_2 production (401.7±95, Mann–Whitney p=0.004 vs. controls) reached values about 30 times higher than control values and 8 times higher than the values at 1 h BD.

Tubular renal injury (Fig. 6.7a,b)
Heart–type fatty acid binding protein (H–FABP), was below detection limits in plasma of all animals, showing minimal release into the circulation of this protein from heart, skeletal muscle, lungs, and brain. H–FABP was also below detection limits in the urine of sham–operated animals. Urine H–FABP concentrations started to rise above detection limits to as early as 0.5 h BD (14.2±5.06 ng/mmol creatinine) and continued to increase after 1 h BD (21.6±5.4 ng/mmol creatinine). Urine H–FABP concentra-

tions at 2 h and 4 h BD were 51.3±7.8 ng/mmol creatinine, and 52.6±8.8 ng/mmol creatinine respectively.

N–acetyl–glucosaminidase (NAG) urine concentrations started to increase non–significantly after 0.5 h of BD, reaching significant higher values 1 h after BD induction (4.2±1 mU/mmol creatinine, Mann–Whitney p=0.01) when compared with control values. Urine NAG continued to increase, reaching the maximum at 4 h BD (2 h BD 6.6±0.2 mU/mmol creatinine, Mann–Whitney p=0.002 vs. controls; 4 h BD 10±2.3 mU/mmol creatinine, Mann–Whitney p=0.002 vs. controls). Urine H–FABP and NAG concentrations correlated significantly (Spearman's correlation coefficient 0.733, sig.<0.001).

6.4 Discussion

The present study demonstrates that brain death induces immediate pro–inflammatory and pro–coagulatory activation of vascular endothelium in rat donor kidneys, which is proportional with the duration of brain death. BD–related donor kidney damage and oxidative stress became subsequently evident, with enhanced injury with prolongation of the BD state. For this study we have used a simple, reproducible and clinical relevant animal brain death rat model in which induction of brain death was obtained by gradual expansion of an intracranial balloon over 30 minutes time period [19]. The model represents an adjustment of brain death models published before by Tilney et al. [20], where the brain death induction was performed over a period of 15 minutes.
The model described here is closely related to the clinical condition of BD due to intracranial haemorrhage, nowadays the most frequent diagnosis of organ donors. This approach stands in contrast to previous studies by our group and by others, where brain death was induced using an explosive onset model with massive brain destruction and critical hypotensive periods reflecting major head trauma. The major benefit of pseudo–stable hemodynamics in the brain death period is that no inotropic medical support is required, which is known to have an effect on organ injury and could bias results in studies concerning brain death related organ damage. Because intracranial hypertension often develops gradually in the clinical setting, we feel that the clinical situation is better represented by this model.

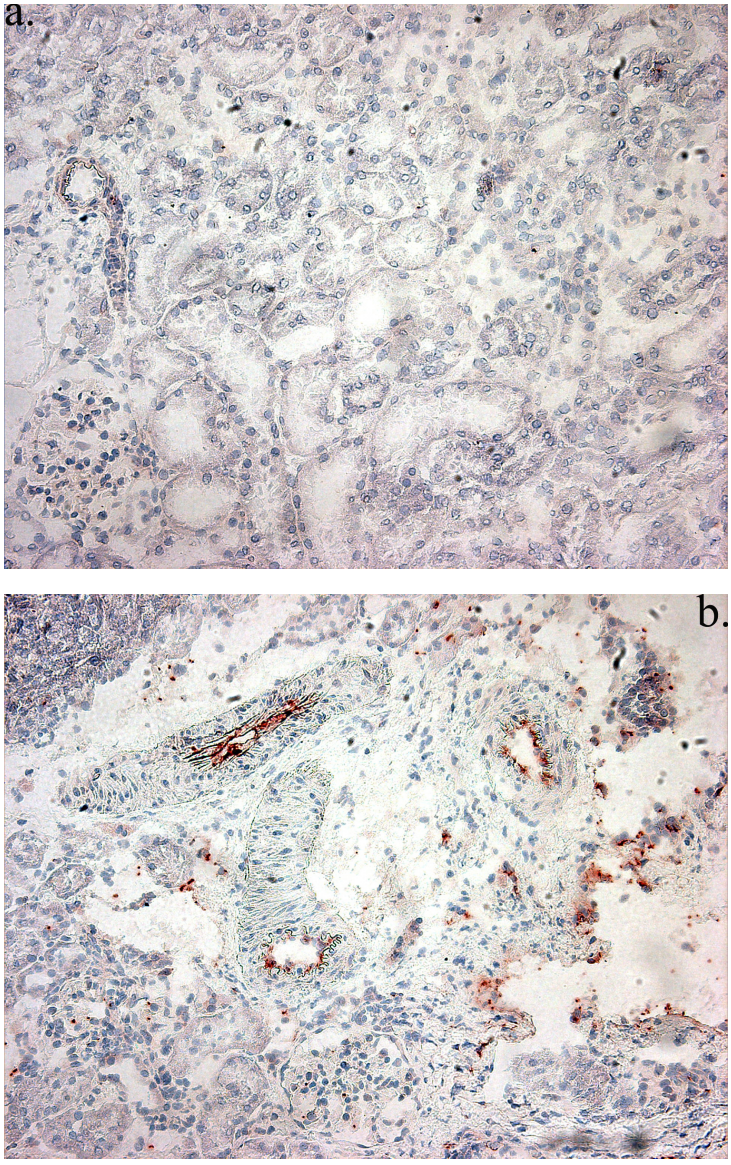

Figure 6.5: Immunohistochemistry using P–Selectin labelled antibody to stain (brown coloration) activated endothelial cells and trapped platelets in rat kidney tissue of a sham–operated animal **(a)** *and a 4 hours brain death animal* **(b)**.

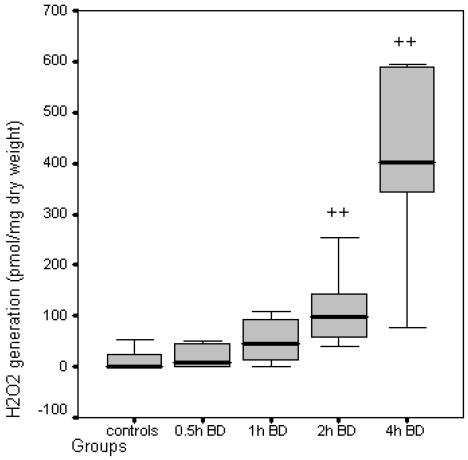

Figure 6.6: Oxidative stress, as quantified by ex–vivo H_2O_2 generation in rat kidney tissue after 0.5, 1, 2, or 4 hours since brain death (BD) induction. The controls are represented by sham–operated animals. Box plots graph data represent statistical values (see Fig. 6.2).

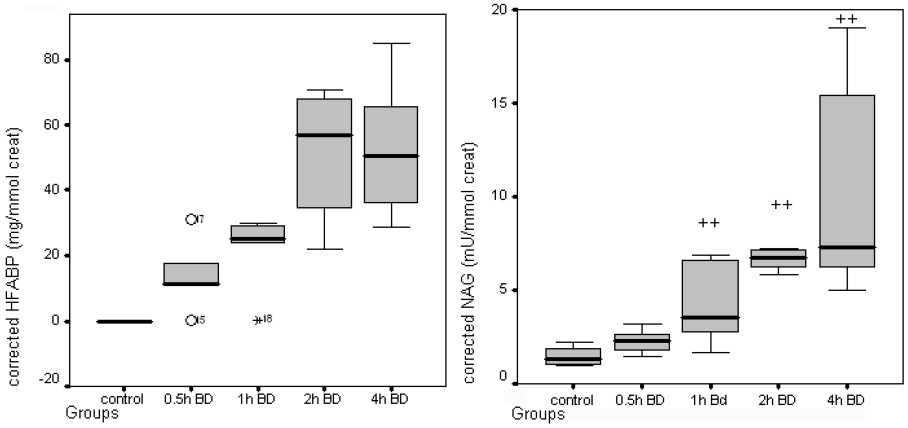

Figure 6.7: Renal tubular injury, as quantified by heart–type fatty acid binding protein (H–FABP, distal tubular injury, **(a)** *and N–acetyl–glucosaminidase (NAG, proximal tubules injury,* **(b)** *in the urine of rats after 0.5, 1, 2, or 4 hours since brain death (BD) induction. The controls are represented by sham–operated animals. Box plots graph data represent statistical values (see Fig. 6.2).*

Hemodynamics
After the onset of brain death the blood pressure decreased to 45 mmHg, most likely due to changes in the vasomotor tone caused by an imbalance between sympathetic and vagal stimulation of the rostral ventrolateral medulla. A gradual increase in blood pressure was observed afterwards, considered a physiological response (Cushing response) to increased intracranial pressure. Subsequently, a peak in blood pressure occurred, followed by a plateau at levels above 100 mmHg. A possible explanation for pseudo–normotension instead of the frequently reported hypotension is that a gradually expanding intracranial mass allows the brain to accommodate, with less distortion in the remaining rostral ventrolateral medulla[21].
Recent studies reported strong evidences of causality between hemodynamic impairment and the systemic inflammatory response. Even if the mechanisms remain unknown, Avlonitis et al.[22] concluded that the sympathetic discharge triggered systemic inflammation, which was further enhanced by neurogenic hypotension.
The endothelial wall has demonstrated abilities to sense small hemodynamic, rheologic and humoral variations with prompt responses to various mechanical (shear stress, viscosity) and inflammatory (interleukins, TNF–α) stimuli. Once activated, the endothelial cells change phenotype to release and synthesize on–demand several vasoactive factors and factors involved in hemostasis and thrombolysis, such as nitric oxide, prostacyclins, vWF, tPA, tissue factor, adhesion molecules and chemoattractant proteins[23]. In our study, we approached endothelial activation by studying the acute release of von Willebrand factor, and expression of E– and P–Selectin. As an additional indicator of increased thrombogenicity we studied the gene expression induction of Aα and Bβ fibrinogen chains. Oxidative stress and kidney injury biomarkers were included in the investigation in order to quantify the clinical relevant end–effect of brain death on organ viability.

Von Willebrand Factor (vWF)
von Willebrand Factor, stored in the endothelial Weibel–Palade storage granules, has unique biomechanical properties and a critical biological role as an adhesive protein; it mediates the adhesion of platelets to injured vascular wall by binding on platelet surface and to collagen in the subendothelium. vWF is one of the most potent activators of platelets, causing them to release additional vWF from their α–storage granules. Increased levels of plasma von Willebrand factor contribute directly to thrombosis, impeding the normal flow of circulating blood[24].
Allograft survival, arteritis and irreversible acute or sub–acute rejection have been reported to be highly associated with intensive staining for vWF on endothelial cells and platelets aggregating in large, medium and small arteries[25]. Furthermore, increased plasma vWF represents a major risk factor for atherosclerosis and vascular disease[26]. It has to be considered as a potential predictor for the development of the alloatherosclerosis of donor organ vessels and chronic rejection through endothelial

injury–induced proliferation of smooth muscle cells.
In our experiment, plasma von Willebrand factor started to rise prominently and significantly two hours after brain death induction, with maximum values after four hours of brain death in this rat model. The elevation in plasma level was significant, reaching levels of more than two times higher than in sham–operated controls.

Fibrinogen
Fibrinogen is a plasma protein whose principal function is exerted through its conversion into soluble fibrin during the process of blood coagulation. In addition, by virtue of its capacity to support platelet aggregation, fibrinogen plays a dual role in thrombus formation. Historically, fibrinogen is known to be synthesized exclusively by hepatocytes and stored in α–granules of megakaryocytes[27,28]. In light of the recently published studies, it is clear that epithelial cells of extrahepatic origin are able to express fibrinogen genes and to secrete intact fibrinogen. Baumheuter et al.[29] found that in addition to being present in the liver, fibrinogen can be expressed on epithelial cells in the kidney, intestine, and spleen. Haidaris[30] demonstrated that, while hepatocytes synthesize and secrete fibrinogen constitutively and on demand, lung epithelial cells synthesize and secrete little intact fibrinogen constitutively. However, after induction with proinflammatory mediators, significant levels of fibrinogen are synthesized and secreted.
Our experiments conclusively demonstrate the expression of both Aα and Bβ fibrinogen chains in the kidneys of brain dead rats. Aα and Bβ fibrinogen chain mRNAs, while poorly expressed in kidneys of sham–operated rats, were abruptly and progressively up–regulated from two hours of brain death onwards, providing evidence that kidneys contribute to changes in acute phase proteins during brain death. The synthesis of fibrinogen in extrahepatic tissue may be triggered in the context of a systemic inflammatory response to brain death. Furthermore, fibrinogen up–regulation might attempt to restore homeostasis by contributing to wound repair or extracellular matrix remodelling after injury.

E– and P–Selectins
E– and P–Selectins belong to the Selectin family of adhesion molecules and play an important role in the inflammatory response, eliciting leukocyte rolling. Both P– and E–Selectins are reported to be critically involved in the early development of acute graft rejection[31]. Up–regulation of gene expression and membrane E– and P–Selectin expression was described before in models of explosive brain death induction, and in recipients of transplanted grafts from brain dead donors[32,33]. A P–Selectin positive expression in a donor biopsy present before transplantation has been shown to predict a high risk of acute rejection[34].
Complementary to the existing data on E– and P–Selectins in brain dead donors, we show an abrupt and progressive up–regulation of E– and P– Selectins starting very

early in the course of brain death, already after half hour from induction and persisting until four hours later. As verified by immunohistochemistry, mRNA up–regulation was closely followed by endothelial cells membrane expression of synthesized selectins. P–Selectin was detected on the surface of endothelial wall starting with half hour of brain death. The intensity and distribution of the staining increased with time, so that in the four hours brain death group P–Selectin was ubiquitously present on the surface of the vascular endothelium. In addition to expression on endothelial cells, P–Selectin labelling revealed the presence of an increased number of platelets in the kidney glomeruli. Since all organs were washed–out consistently and in a standardized manner, a progressively increasing number of platelets in time might reflect enhanced platelet adhesion to the vascular endothelium. These findings are consistent with the data showing increased plasma vWF levels in brain dead rats, a factor known to support platelet adhesion to the vascular wall.

Clinical and experimental studies investigating the therapeutic role of administrating recombinant proteins targeted against P–Selectin showed a marked improvement in graft outcome, blocking neutrophil and lymphocyte infiltration and thus decreasing inflammatory response and ischemia–reperfusion injury[35,36]. Different therapeutic approaches were studied, administrating the P–Selectin blocker to the donor (after 6 h BD–kidneys perfused in situ, or 3 h BD–intravenous injection) or to both donor and recipient (after 3 h BD intravenous injection to the donor and at the time of reperfusion in the recipient). The results of the present investigation show E– and P–Selectin up–regulation and expression already after half hour from brain death declaration, pointing out the need to introduce blocker therapy a lot earlier in the course of brain death donation.

Oxidative stress

Different authors have found reactive oxygen species (ROS) formation to be a reliable predictor of allograft rejection, especially in the early ischemic post–transplant period[37]. High levels of ROS are also known to contribute to the development of atherosclerosis of organ donor vessels and chronic rejection through endothelial–injury–induced proliferation of smooth muscle cells[38].

In our approach, we have tested ex–vivo the mitochondrial (dys)function in kidney tissue of brain dead animals, as reflected by ROS–production when oxidizing pyruvate and succinate. General evidence for the source of ROS production points at dysfunctional mitochondria in cells pre–exposed to hypoxia–reoxygenation, activated endothelial cells and infiltrated inflammatory cells[39–41]. The production of superoxide and hydroxyl radicals, measured as H_2O_2 generation, started to increase one hour after BD induction, reaching levels significantly higher only two hours after brain death induction, and maximum values after four hours. It is important also to analyze these results in the time sequence presented here: oxidative stress arises only after the pro–inflammatory response during brain death. This observation might solve a very

important mechanistic issue – it is the inflammatory response and not the hypoxia that is triggered first after brain death!

Kidney injury (bio)markers
To date, sensitive and specific biomarkers to assess donor organ viability are lacking. Non–invasive and easy accessible biomarkers assessing renal tubular injury are heart–type fatty acid binding protein (distal tubular injury) and N–acetyl–glucosaminidase (proximal tubular injury). Both markers were included in this study to investigate BD–derived renal damage.
Heart–type fatty acid binding protein (H–FABP) is a cytosolic protein abundant in the myocardium, but also expressed in kidneys (distal tubules), skeletal muscles, lung, and brain[42]. H–FABP has been associated before with early release following injury of distal renal tubules[43].
The levels below detection limit of this marker we found in the plasma of all animals proved a primary release of urinary H–FABP from kidneys, with minimal release of this protein from heart, skeletal muscle, lungs or brain in this model.
Urine N–acetyl–glucosaminidase (NAG) release signifies renal damage localized at the level of proximal tubules. Urinary NAG has been measured in renal transplant recipients soon after transplantation to predict acute rejection or chronic allograft nephropathy[44].
In our study, H–FABP and NAG urine concentrations reached significantly higher values as early as half hour and one hour, respectively, after brain death induction, when compared with sham–operated animals. A highly positive correlation was documented between the two renal tubule markers, suggesting a similar pathophysiologic mechanism and consolidating the diagnose of renal tubular damage during brain death. Both proximal and distal renal tubules injuries were early diagnosed in the course of brain death, even before an oxidative hypoxic stress (as shown by H_2O_2 generation) took place. The trigger responsible for this early organ injury might be represented by the inflammatory response, shown in our experiments to arise immediately after brain death induction. The values continued to increase progressively during the studied period, which points at an enhanced loss of organ viability with prolongation of the state of brain death.

In summary, this study demonstrates immediate pro–inflammatory and pro–coagulatory activation of vascular endothelium after BD in kidney donor rats, proportional with the duration of BD. The mRNA expression of the adhesion molecules E– and P–Selectins, known to promote inflammation by mediating rolling and extravasation of leukocytes, were up–regulated soon (half hour) after brain death induction. Additionally, platelet trapping, most probably due to platelet adhesion to the vascular wall, was visualized as early as half hour after inducing the brain death. To support arguments for an increased thrombogenicity status in brain dead donors, we

report a significant increase in plasma levels of von Willebrand factor, that reflects sustained platelet adhesion to the vascular wall. Additionally we show an increased mRNA expression of Aα and Bβ fibrinogen, which will promote extracellular matrix remodelling and thrombogenesis by forming the fibrin network and mediating platelet adhesion. We found for the first time that the brain death related systemic inflammatory response induces an extrahepatic Aα and Bβ fibrinogen synthesis. Oxidative stress started to increase after induction of brain death, and became significant only after two hours of BD. Our data point at an ischemic/hypoxic oxidative stress, as main cause of oxygen radicals production, during protracted periods of brain death. BD–related donor kidney damage, reflected by urine concentration of heart–type fatty acid binding protein and N–acetyl–glucosaminidase, was diagnosed as early as half hour in renal tubules, with enhanced loss of viability when the state of brain death was prolonged.
These data suggest that early anti–inflammatory and anti–coagulatory therapeutic intervention should be instituted after declaration of brain death, aiming to reduce endothelial activation, prevent platelet adhesion, and possibly slow down an ongoing donor organ deterioration.
Our study also indicates that without effective cytoprotective agents, organ retrieval should not be postponed longer than absolutely necessary, to prevent further injury and loss of viability of scarce donor organs.

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References

1. Power BM, Van Heerden PV. The physiologic changes associated with brain death-current concepts and implications for the treatment of the brain dead organ donor. *Anaesth. Intensive Care.* 1995;23:26-36.

2. Novitzky D, Cooper DK, Reichart B. Hemodynamic and metabolic responses to hormonal therapy in brain dead potential organ donors. *Transplantation.* 1987;43:852-5.

3. Bittner HB, Chen EP, Kendall SW, Van Trigt P. Brain death alters cardiopulmonary hemodynamics and impairs right ventricular power reserve against an elevation of pulmonary vascular resistance. *Chest.* 1997;111:706-11.

4. Lelkes PI. Mechanical Forces and the Endothelium. *London: Harvard Academic.* 1999;33-54.

5. Ali MH, Schumacker PT. Endothelial responses to mechanichal stress: Where is the mechanosensor? *Crit. Care Med.* 2002;30:S198-S206.

6. Fisher AB, Chien S, Barakat AI, Nerem RM. Endothelial cellular response to altered shear stress. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2001;281:L529-33.

7. Nilius B, Droogmans G. Ion Channels and their functional role in vascular endothelium. *Physiol. Rev.* 2001;81:1415-9.

8. van der Hoeven JA, Ploeg RJ, Postema F, Molema I et al. Induction of organ dysfunction and up-regulation of inflammatory markers in the liver and kidneys of hypotensive brain dead rats: a model to study marginal organ donors. *Transplantation.* 1999;68:1884-90.

9. Schuurs TA, Gerbens F, van der Hoeven JA, Ottens PJ et al. Distinct transcriptional changes in donor kidneys upon brain death induction in rats: insights in the processes of brain death. *Am. J. Transplant.* 2004;4:1972-81.

10. Skrabal CA, Thompson LO, Potapov EV, Southard RE et al. Organ-specific regulation of pro-inflammatory molecules in heart, lung, and kidney following brain death. *J. Surg. Res.* 2005;123:118-25.

11. van Der Hoeven JA, Ter Horst GJ, Molema G, de Vos P et al. Effects of brain death and hemodynamic status on function and immunologic activation of the potential donor liver in the rat. *Ann. Surg.* 2000;232:804-13.

12. Wilhelm MJ, Pratschke J, Beato F, Taal M, et al. Activation of the Heart by Donor Brain Death Accelerates Acute Rejection After Transplantation. *Circulation.* 2000;102:2426.

13. Pou S, Huang YI, Bhan A, Bhadti VS et al. A fluorophore-containing nitroxide as a probe to detect superoxide and hydroxyl radical generated by stimulated neutrophils. *Anal. Biochem.* 1993;212:85-90.

14. Li B, Gutierrez PL, Blough NV. Trace determination of hydroxyl radical in biological systems. *Anal. Chem.* 1997;69:4295-302.

15. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL. Production of reactive oxygen species by mitochondria: central role of complex III. *J. Biol. Chem.* 2003;278:36027-31.

16. Li B, Gutierrez PL, Blough NV. Trace determination of hydroxyl radical using fluorescence detection. *Methods Immunol.* 1999;300:202-216.

17. Harboe A. Method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry. *Scand. Clin. Lab. Invest.* 1959;11:66-70.

18. Lockwood TD, Bosmann HB. The use of urinary N-acetyl-β-glucosaminidase in human renal toxicology: I. Partial biochemical characterization and excretion in humans and release from the isolated perfused rat kidney. *Toxicol. Appl. Pharmacol.* 1979;49:323-36.

19. Kolkert JLP, 't Hart NA, Van Dijk A, Ottens PJ, Ploeg RJ, Leuvenink HGD. A new gradual onset brain death model in rat for studies concerning organ donation. Submitted to *Am. J. Transplant.*

20. Gasser M, Waaga AM, Kist-Van Holthe JE, Lenhard SM, Laskowski I, Shaw GD, Hancock WW, Tilney NL. Normalization of brain death-induced injury to rat renal allografts by recombinant soluble P-selectin glycoprotein ligand. *J. Am. Soc. Nephrol.* 2002;13:1937-45.

21. Dampney RA, Horiuchi J, Tagawa T, Fontes MA et.al. Medullary and supramedullary mechanisms regulating sympathetic vasomotor tone. *Acta Physiol. Scand.* 2003;177:209-18.

22. Avlonitis VS, Wigfield CH, Kirby JA, Dark JH. The hemodynamic mechanisms of lung injury and systemic inflammatory response following brain death in the transplant donor. *Am. J. Transplant.* 2005;5:684-93.

23. Cines DB, Pollak ES, Buck CA, Loscalzo J et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood.* 1998;91:3527-61.

24. Ruggeri ZM. Role of von Willebrand factor in platelet thrombus formation. *Ann. Med.* 2000;32:2-9.

25. Ota H, Fox-Talbot K, Hu W, Qian Z et al. Terminal complement components mediate release of von Willebrand factor and adhesion of platelets in arteries of allografts. *Transplantation.* 2005;79:276-81.

26. Blann AD. Endothelial cell activation, injury, damage and dysfunction: separate entities or mutual terms? *Blood Coagul Fibrinolysis* 2000;11:623-630.

27. Leven RM, Schick PK, Budzynski AZ. Fibrinogen biosynthesis in isolated guinea pig megakaryocytes. *Blood.* 1985;65:501.

28. Louache F, Debili N, Cramer E, Breton-Gorius J et al. Fibrinogen is not synthesized by human megakaryocytes. *Blood.* 1991;77:311-6.

29. Baumhueter S, Mendel DB, Conley PB, Kuo CJ et al. HNF-1 shares three sequence motifs with the POU domain proteins and is identical to LF-B1 and APF. *Genes Dev.* 1990;4:372-9.

30. Haidaris PJ. Induction of fibrinogen biosynthesis and secretion from cultured pulmonary epithelial cells. *Blood.* 1997;89:873-82.

31. Yamazaki S, Isobe M, Suzuki J, Tojo S et al. Role of selectin-dependent adhesion in cardiac allograft rejection. *J. Heart Lung Transplant.* 1998;17:1007-16.

32. Takada M, Nadeau KC, Hancock WW, Mackenzie HS et al. Effects of explosive brain death on cytokine activation of peripheral organs in the rat. *Transplantation.* 1998;65:1533-42.

33. Pratschke J, Kofla G, Wilhelm MJ, Vergopoulos A, et al. Improvements in early behavior of rat kidney allografts after treatment of the brain-dead donor. *Ann. Surg.* 2001;234:732-40.

34. Benson SR, Ready AR, Savage CO. Donor platelet and leukocyte load identify renal allografts at an increased risk of acute rejection. *Transplantation.* 2002;73:93-100.

35. Farmer DG, Shen XD, Amersi F, Anselmo D et al. CD62 blockade with P-Selectin glycoprotein ligand-immunoglobulin fusion protein reduces ischemia-reperfusion injury after rat intestinal transplantation. *Transplantation.* 2005;79:44-51.

36. Gasser M, Waaga-Gasser AM, Grimm MW, Grimm MR et al. Selectin blockade plus therapy with low-dose sirolimus and cyclosporin a prevent brain death-induced renal allograft dysfunction. *Am. J. Transplant.* 2005;5:662-70.

37. Kosieradzki M, Kuczynska J, Piwowarska J, Wegrowicz-Rebandel I et al. Prognostic significance of free radicals: mediated injury occurring in the kidney donor. *Transplantation.* 2003;75:1221-7.

38. Land W. The potential impact of the reperfusion injury on acute and chronic rejection events following organ transplantation. *Transplant. Proc.* 1994;26:3169-71.

39. Terada LS. Hypoxia-reoxygenation increases O2 efflux, which injures endothelial cells by an extracellular mechanism. *Am. J. Physiol. Heart Circ. Physiol.* 1996;270:H945-H950.

40. Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am. J. Physiol. Cell Physiol.* 2002;282:C227-41.

41. Baud L, Ardaillou R. Reactive oxygen species: production and role in the kidney. *Am. J. Physiol.* 1986;251:F765-76.

42. Veerkamp JH, Paulussen RJ, Peeters RA, Maatman RG et al. Detection, tissue distribution and (sub)cellular localization of fatty acid-binding protein types. *Mol. Cell Biochem.* 1990;98:11-8.

43. Gok MA, Pelzers M, Glatz JF, Shenton BK et al. Do tissue damage biomarkers used to assess machine-perfused NHBD kidneys predict long-term renal function post-transplant? *Clin. Chim. Acta.* 2003;338:33-43.

44. Bone JM, Amara AB, Shenkin A, Hammad A et.al. Calcineurin inhibitors and proximal renal tubular injury in renal transplant patients with proteinuria and chronic allograft nephropathy. *Transplantation.* 2005;79:119-22.

Chapter 7

Organ Perfusion During Organ Transplantation: Consequences and Importance of the Washing Out Procedure.

Hyperaggregating Effect of HES Components and University of Wisconsin Solution on Human Red Blood Cells: A Risk of Impaired Graft Perfusion in Organ Procurement?

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Abstract

Study Objectives

To date, the standard preservation solution used during organ procurement and preservation of most organs is the University of Wisconsin (UW) solution. Despite its superiority over other cold storage solutions, the inclusion of hydroxyethyl starch (HES) as one of the components of the UW solution has been both advocated and denied. The aim of this study was to determine the effect of HES on red blood cell aggregability and to correlate aggregation parameters with HES molecular weight.

Methods

Human RBC aggregability and deformability were investigated in vitro, at 4° C, with a Laser–assisted optical rotation cell analyzer. The study of red blood cell aggregation in a binary HES–HES system gave an indication about the nature of HES–RBCs interactions. Bright field microscopy and atomic force microscopy were used to morphologically characterize the aggregates size and form.

Results

High molecular weight HES and UW solution had a potent *hyper*-aggregating effect; low molecular weight HES had a *hypo*-aggregating effect on RBC. RBC aggregates were of large size and their resistance to dissociation by flow induced shear stress was high.

Conclusions

Our in vitro experiments conclusively showed that the physiological function of red blood cells to form aggregates is significantly affected in the presence of hydroxyethyl starch. The use of high molecular weight HES in UW solution accounts for extended and accelerated aggregation of erythrocytes that may result in stasis of blood and incomplete wash–out of donor organs prior to transplantation.

7.1 Introduction

Reperfusion injury after cold ischemic storage prior to organ transplantation plays a critical role in the occurrence of primary nonfunction and delayed graft function[1] which have remained major problems in liver transplantation[2]. The viability of organ grafts depends on several factors such as cold ischemia time, the perfusion procedure, preservation methods and reperfusion quality. The efficacy of perfusion during the initial wash–out procedure, however, has to be considered a major determinant of functional recovery after transplantation[3].

Preservation solutions have been designed to ameliorate the adverse physiological and biochemical effects of ischemia under hypothermic conditions. Three principles are important in effective cold storage. First, the vascular wash–out during harvest should rapidly cool the organs, remove the blood and allow balance between the cold storage solution and the tissue. Second, the cold storage solution should prevent cell swelling and interstitial edema formation by including substances that are osmotically active and impermeable to the cell. Impermeants and saccharides achieve homeostasis of the intracellular water content. Homeostasis of the interstitial compartments is achieved by counteracting a hydrostatic force during the initial wash–out using colloids. The intravascular fluid compartment does not need an effective component in static cold–storage. Third, the cold storage solutions should prevent excessive cellular acidosis by containing sufficient concentration of hydrogen–ion buffer, histidine or citrate[4,5].

Since its introduction by Belzer et al. in the late eighties, the University of Wisconsin (UW) solution has become the standard solution for the preservation of most organs in transplantation. Despite the fact that UW solution made extended cold preservation feasible, some studies have demonstrated that prolonged cold ischemic time of hepatic allografts enhance bacterial infection[6], cause biliary and hepatic artery complications[7,8] and increase the frequency of primary non function posttransplant[9].

The inclusion and importance of the colloid hydroxyethyl starch (HES) as one of the components of the UW solution has been both advocated and denied. HES prevents interstitial edema and has a beneficial effect on matrix metallo–proteinases[10] but at the price of a higher solution viscosity. Due to the presence of HES, the viscosity of UW solution at 4° C increased by a factor of 2.5 when compared with the viscosity of the same solution at 37° C[11]. Analyzing the effect of HES on the rheological properties of blood, Corry and collaborators have drawn the attention to the aggregating effect of HES on RBC[12].

The pathogenic potential of RBC aggregates prevails within the microcirculation, leading to altered flow dynamics and microvessel occlusive events[13,14]. Furthermore, cell–cell interaction between platelets and erythrocytes can significantly enhance platelet reactivity with a prothrombotic effect[15,16]. There is also strong evidence that RBC aggregation greatly enhances the tendencies of leukocytes to adhere to the postcapillary endothelium, a process recognized as essential in inflammation[17]. Considering these aspects, HES induced–RBC aggregability could significantly influ-

ence the quality of organ preservation, increase damage due to ischemia/reperfusion and affect the outcome after transplantation.
This study concerns an extension to a previous observation made by our group [18] that signalled a poor initial wash–out of rat liver when using UW solution. We concluded at that time that this effect is most likely the consequence of aggregate–formation induced by HES in combination with rat blood. The present study will reveal a detailed evaluation of the extent and kinetics of HES–induced human RBC aggregation, as well as a morphological characterization of these aggregates. In addition, to explain the mechanisms involved, red blood cell aggregation has been studied in a binary HES–HES mixture.

7.2 Methods

RBC aggregability and deformability were investigated in vitro with a Laser–assisted optical rotation cell analyzer (LORCA R&R Mechatronics, Hoorn, The Netherlands) [19,20]. This instrument, based on the ektacytometric principle, is equipped with a video camera for detection of the laser diffraction pattern, a thermostation unit and an ellipse–fit computer program calculating the Elongation Index and Aggregation Index (AI). The experiments were performed at 4° C, after in vitro admixture of UW/HES solutions with human fresh blood from healthy volunteers (n=8), drawn from the antecubital vein into 0.1 mM ethylenediamine tetracetic acid.
Three commercially available HES solutions (6% in 9 g/l sodium chloride) were selected based on their molecular weight and substitution ratio: HES 450/0.7 (M_w=450 kDa, MS=0.7), HES 200/0.5 (M_w=200 kDa, MS=0.5), HES 130/0.5 (M_w=130 kDa, MS=0.5). Phosphate Buffered Saline (PBS, pH 7.4, 300 mOsm/kg) was used as buffer fluid for the HES solutions. The final concentration of the HES solutions was 5%, pH=7.4, isotone. The University of Wisconsin solution was used as commercially available (5% HES with a molecular weight cut off range of 100–1000 kDa and a mean of 250 kDa). As a negative control, we tested the effect of a HES–free UW solution (prepared according to the UW–recipe without the addition of HES).
The samples were prepared not more than one hour before the measurements took place, the mixing ratios were: blood:HES = 5:1, 7:1, 10:1; blood:UW / HES–free UW = 5:1 , 2:1. The hematocrit (Hct) was adjusted in all the samples to a constant value of 38%. A control (red blood cells suspended in autologous plasma, 38% Hct) was considered for every set of samples.
Aggregation of human red blood cells in binary HES–HES mixtures, a competitive assay: human erythrocytes were treated (as previous) with HES 450 kDa and HES 130 kDa solutions, using a mixing ratio of 5:1. After measuring the effect on the aggregation, HES 130 kDa was added on the HES 450 kDa–treated samples and vice–versa, to a final mixing ratio of 5:2. The experiment was performed at room temperature

(22° C).
For the evaluation of RBC aggregation we recorded several comparative parameters.

LORCA Measurements

For the determination of red cell aggregation, the blood is brought under a shear rate of $500\,s^{-1}$, after which the shear is stopped at t=0. The backscattered intensity from the blood layer is measured during 120 s after shear stop. The intensity drops because of red blood cell aggregation. We considered the beginning point of the aggregation the extrapolated value of the decay curve towards t = 0. The kinetics of the aggregation was studied using two parameters: *aggregation index and the half time* ($T_{1/2}$ = the time necessary to reach 50% aggregation). *The minimal value of shear rate that prevents aggregation* gave an indication of the strength of the intercellular interaction by determining the aggregates' resistance to dissociation by flow–induced shear stress.
The deformability of the red cells has been determined by repeatedly measuring the diffraction pattern of red cells under various shear stresses in the range of 0.01–100 Pa, from which an Elongation Index has been calculated by LORCA software. The blood was diluted 200 times in PolyVinylPyrolidone (5 g/l) in PBS (50 mM).

Viscosity Measurements

For the measurements of the viscosity of blood and HES–treated blood we used an automated dynamic shear rheometer with cone–plate geometry (AR1000 Rheometer, TA Instruments). During measurements the temperature was set at 4° C and the shear rate of operation equaled the value of the corresponding shear rate which prevented aggregation for that specific sample.

Imaging Techniques

Light Microscopy and Atomic Force Microscopy were used to morphologically characterize the aggregates' size and form.
Bright field microscopy provided a direct, large scale, two–dimensional visualization of the samples. Images were digitized and statistics of the aggregates' area size were generated using the Image Pro–Plus software (version 3.0.1 Media Cybernetics).
Tapping–Mode Atomic Force Microscopy provided three–dimensional imaging of unstained and uncoated RBC aggregates in air. Sample preparation consisted of a standard smear of 300 times diluted filtered blood on a glass surface. In this way, sample preparations and imaging environments known to generate artifacts are eliminated (e.g., dehydration, fixation, freezing, staining and coating).

Statistical Analysis

Differences between physiological and experimental aggregation parameters in different samples groups were evaluated using the paired two tailed Student T Test. A p value of < 0.05 was considered statistically significant. The results are expressed as mean±SD.

7.3 Results

LORCA Measurements

The molecular weight of HES had a highly significant influence on the *kinetics of RBC aggregation* (Fig. 7.1a). For a blood:HES ratio of 5:1 the aggregation index in the presence of HES 450 kDa was 39.76±5.99, an increase of more than 100% as compared to the control aggregation index, 18.16±3.43, ($p<0.01$). In contrast, the low molecular weight HES significantly reduced the RBC aggregability ($p=0.019$); the AI measured in the HES 130 kDa treated samples was 13.64±1.96. In addition, we determined the concentration–dependent effects of HES on RBC aggregation. Decreasing HES 450 kDa and HES 200 kDa concentrations resulted in a concomitant decrease of aggregation index, although 10% HES 450 kDa still induced a significant increased aggregation ($p<0.01$).

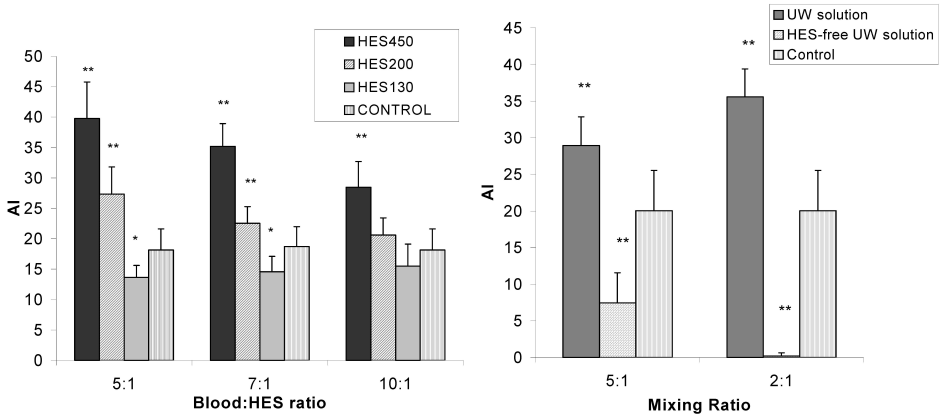

Figure 7.1: **(a)** *Aggregation Index (AI) of human blood treated with different HES in various ratios (mean±SD).* **(b)** *Aggregation Index (AI) of human blood treated with UW solution/HES–free UW solution using different mixing ratios (mean±SD). Highly significant (** $p<0.01$) and significant (* $p<0.05$) differences with whole blood are indicated.*

	Shear rate (s^{-1}) mean±SD	Viscosity (mPa.sec) mean±SD	Shear stress (Pa) mean±SD
UW solution–treated samples	175±29	14.9±0.3	2.5±0.2 **
HES 450 kDa treated samples	240±70	12.4±0.2	3.4±0.2 **
HES 200 kDa treated samples	140±12	15.1±0.5	2.0±0.1 **
HES 130 kDa treated samples	86±15	18.2±0.5	1.5±0.05 **
Controls	78±3	22.7±0.5	1.6±0.1

*Table 7.1: The shear stress required to dissociate RBC aggregates. Highly significant ($p<0.01$) differences as compared with whole blood samples (controls) are indicated with **.*

The AI measured in the UW treated blood was 28.94±3.89 for the ratio 5:1 and 35.55±3.84 for the ratio 2:1; the control sample had an AI of 20.02±5.52. When treating the blood with colloid free–UW solution in a ratio of blood:HES–free UW solution = 2:1, the aggregation index decreased to 0.20±0.42 (Fig. 7.1b).
The kinetics of the aggregation process is also expressed by the half–time ($T_{1/2}$) value. Since $T_{1/2}$ is the time necessary to reach 50% of complete aggregation level, a lower $T_{1/2}$ reflects a faster aggregation process. The RBC aggregates formed three times faster when the cells came in contact with HES 450 kDa ($T_{1/2}$=6.67±0.84 s), as compared to the control ($T_{1/2}$=20.43±4.59 s) ($p<0.01$). HES 130 kDa inhibited the aggregation process, the half time necessary for RBC treated with HES 130 kDa to reach complete aggregation ($T_{1/2}$=29.17±6.68 s) was significantly higher ($p=0.024$) when compared to control.
Resistance to dissociation by flow induced shear stress expresses the strength of the aggregates. The shear stress required to dissociate the aggregates is calculated by multiplying the minimum shear rate that prevents aggregation with the viscosity of the blood at that shear rate:

$$\text{Shear Stress (mPa)} = \text{Shear Rate } (s^{-1}) \times \text{Viscosity (mPa.s)}$$

The measured shear rates that prevented aggregation, the viscosity values measured for each sample at the corresponding shear rate, and the calculated shear stresses are presented in Table 7.1. It was notable that the viscosity values of the control were higher than the viscosity measured for the HES 130 kDa treated samples, the conditions of the measurement being the same (shear rate = 80 s^{-1}, temperature 4°C).
Erythrocyte deformability measured by means of Elongation Index parameter with LORCA, showed no significant differences between HES treated samples and control samples.
Aggregation of human red blood cells in binary HES–HES mixtures, a competitive assay: for red blood cells pretreated with HES 450 kDa, aggregation index decreased

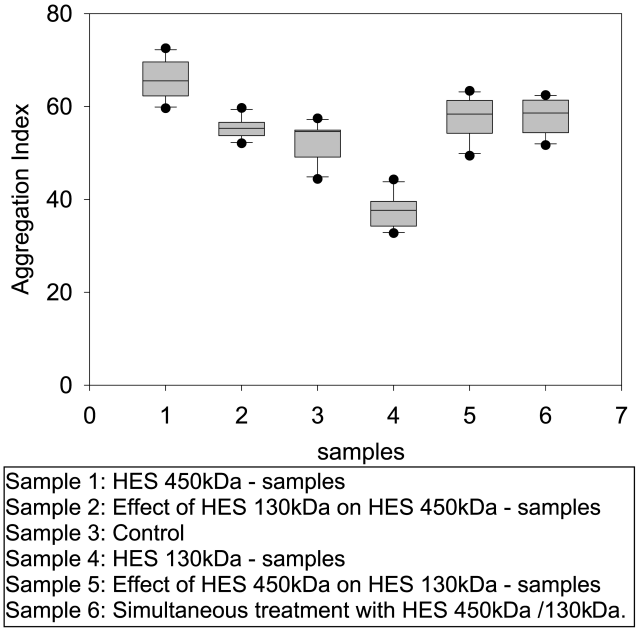

Figure 7.2: Aggregation of human red blood cells in binary HES–HES mixtures, a competitive assay. Box plots graph data represent statistical values. The boundary of the box closest to zero indicates the 25$^{\text{th}}$ percentile, the line within the box marks the median of 6 measurements, and the boundary of the box farthest from zero indicates the 75$^{\text{th}}$ percentile. Whiskers above and below the box indicate the 90$^{\text{th}}$ and 10$^{\text{th}}$ percentiles.

when adding small starch (from 65,8±4,7 to 55,4±2,6). For red blood cells pretreated with HES 130 kDa, large HES increased the aggregation index from 37,6±4,1 to 57,5±5,2. Concomitant adding of HES 450 kDa and HES 130 kDa to the red blood cell suspension yielded values similar to those obtained by consecutive treatment with HES 450 kDa and HES 130 kDa (Fig. 7.2).

Imaging Techniques

The large–scale light microscopic images showed clear differences between the extent of aggregation in the HES–treated samples and the control samples. The statistics on these images, given by Image Pro–Plus software are shown in Table 7.2.
The UW solution treatment of the RBC determined formation of branched rouleaux networks with a range of 23–56 cells per aggregate (Fig. 7.3a). HES 450 kDa induced formation of large size RBC aggregates with an irregular geometry: polymorph ery-

throcyte clusters were clearly visualized (Fig. 7.3b). The morphology of the HES 130 kDa induced RBC aggregates consisted of various size linear rouleaux (Fig. 7.3c). The image of the erythrocytes treated with HES–free UW solution confirmed the absence of RBC aggregation; at a magnification of 200× only 8 aggregates were counted, with a range of 2–3 cells per aggregate. A control was considered as well (Fig. 7.3d). Tapping–Mode atomic force microscopy technique revealed a three dimensional surface profile of RBC aggregates with micrometer resolution. This visualization approach provided clear evidence of aggregation between intact red blood cells when treated with high molecular weight hydroxyethyl starch/UW solution (Fig. 7.4a,b, respectively).

Table 7.2: Statistics given by Image Pro–Plus Software after processing bright field microscopy images taken at a magnification of 200×.

	HES 450 kDa	HES 130 kDa	UW solution	HES – free UW	CONTROL
Total cell count	1881	1289	1545	349	164
Total aggregate count	154	216	64	8	4
Single cells (%)	4.9	35	2.9	95.1	94.5
Cells in aggregate (%)	95.1	65	97.1	4.9	5.5
Cells / aggregate (range)	10–28	4–7	23–56	2–3	2–3
Area Max. (μm^2)	6740	1398	4332	72	137

7.4 Discussion

In the present study, we conducted a comparative analysis of various parameters expressing the aggregation status of RBC in samples treated with University of Wisconsin solution and different molecular weight HES solutions. Our findings indicate that high molecular weight hydroxyethyl starch solutions (HES 450 kDa and HES 200 kDa) as well as UW solution have a potent hyperaggregating effect on human RBC. RBC aggregates formed in the presence of this colloid are of large size; the maximum size aggregate area was 6740 μm^2 in the HES 450 kDa treated samples and 4332 μm^2 in the UW–treated samples. In addition, their resistance to dissociation by flow induced shear stress is increased by 50–100% compared to control samples. These data suggest that gravity–induced hydrostatic perfusion pressures presently used in procurement can not easily dissociate the abnormal RBC aggregates. Some authors have advocated a more physiologic method in which the UW solution is flushed under pressure (100 mmHg) similar to the mean arterial blood pressure with the advantage of perfusing the small intrahepatic vessels. Measurement of the microvascular blood flow patterns in physiologic conditions using intravital microscopy shows that in ar-

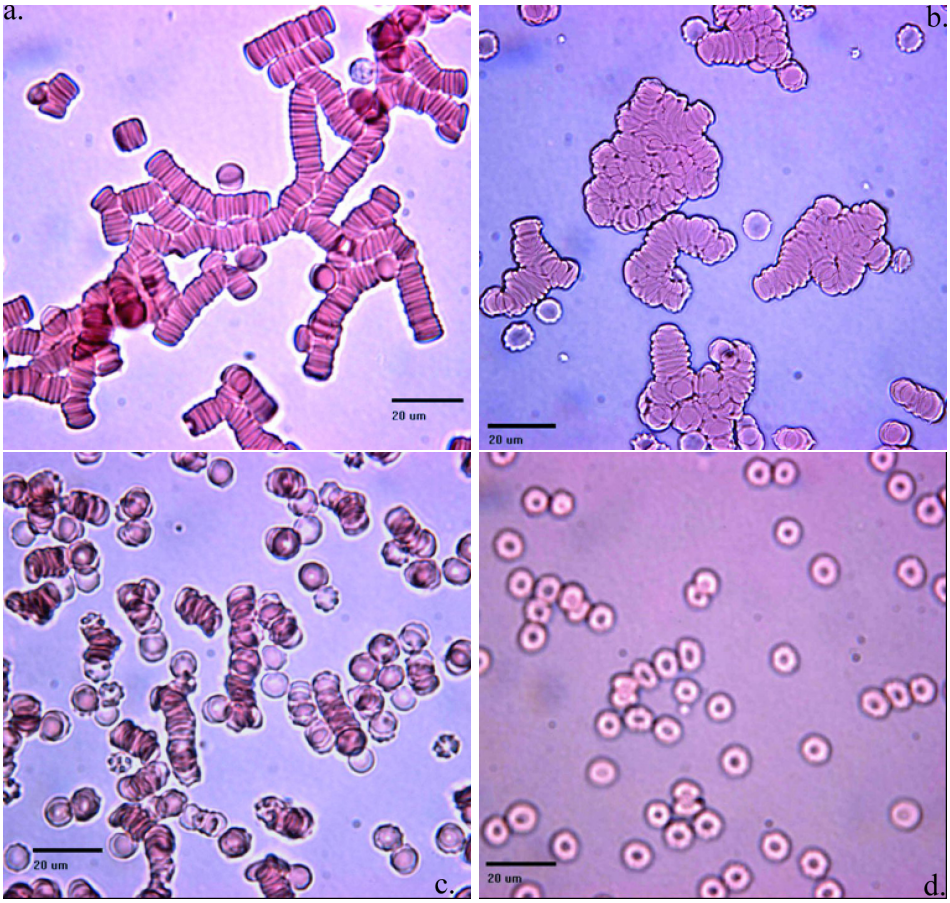

Figure 7.3: Bright field microscopy, magnification 500×. Bar scale represents 20 μm. **(a)** *UW–induced branched RBC rouleaux networks,* **(b)** *HES 450 kDa induced RBC polymorph clusters,* **(c)** *HES 130 kDa induced linear RBC rouleaux,* **(d)** *Control–RBC suspended in autologus plasma.*

terioles and venules, with a diameter of 24.7±9.1 μm, the recorded shear rate has a mean value of 201±163 s^{-1} [21]. The minimal value of the shear rate that prevented UW–induced aggregation in our experiments was 175±29 s^{-1}. These data indicate that even with a high–pressure perfusion, the low shear rates generated in certain areas and the small vessel diameter compared to the aggregates size make this vessel category prone to mechanical obstruction. In addition, increasing the perfusion pressures could represent and additional stress factor for sinusoidal endothelial cells.

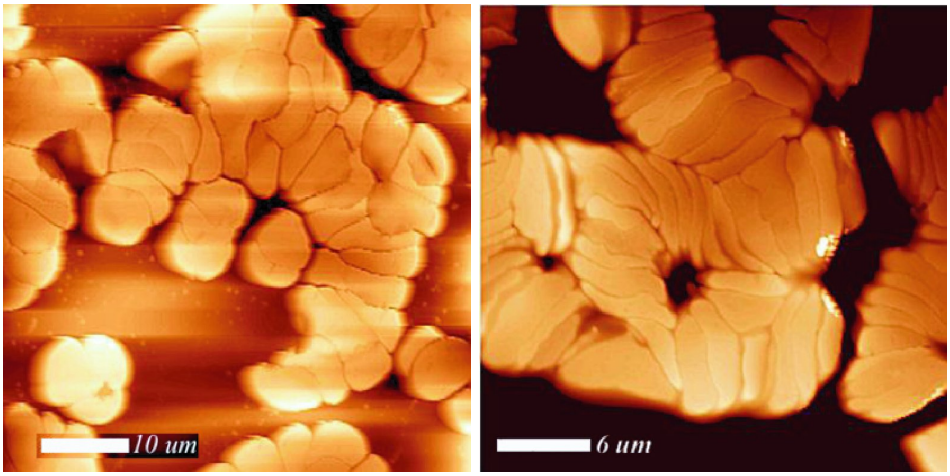

Figure 7.4: Tapping–Mode Atomic Force Microscopy. **(a)** *HES 450 kDa treated red blood cells cluster of 44.18 µm/53.35 µm with irregular geometry (scan size 60 µm/60 µm),* **(b)** *Branched RBC rouleaux network induced by UW solution – detailed topography (scan size 30 µm/30 µm).*

These cells are already particularly vulnerable to cold ischemia/reperfusion injury and thus are believed to be the primary target of this injury[22].
The presence of remaining host erythrocyte aggregates after the initial wash–out of the donor organ could contribute to an inadequate microvascular perfusion with preservation solution and therefore to a poor maintenance of graft viability during ischemic storage. The areas of the respective organs that are only marginally equilibrated with University of Wisconsin solution are less protected during the subsequent ischemic storage period, thus contributing to an overall reduced structural and functional integrity of the organ[23]. Preservation injuries are considered to be major contributors to primary allograft failure after liver transplantation. Inadequate preservation with UW solution for 16 hours becomes histologically evident 24 hours after reperfusion: submassive confluent necrosis of hepatocytes associated with loss of intercellular borders mainly in the midzonal region, with selective sparing of periportal and centrilobular zones[22]. In this respect, Busquets et al. reported the presence of preservation injuries in 17% of the liver grafts preserved in UW solution and associated the presence of these lesions with an increase of posttransplant biliary complications[24].
In addition, mobilization of resting host red blood cells during reperfusion time, the presence of lysed erythrocytes and endothelial cells due to cold ischemia and inadequate microvascular perfusion with preservation solution may lead to a local hypercoagulable state. Local activation of the coagulation system on graft reperfusion may

cause intravascular and/or intracardiac thrombus formation and pulmonary thromboembolism[25]. Suriani et al. suggested that subclinical thromboembolism on graft reperfusion is common. He reported echodense masses in the right atrium within one min after reperfusion in 70% of the patients undergoing liver transplantation[26]. Thus, it could be possible that by identifying the RBC hyperaggregating effect of UW solution as an etiology–related factor for these complications immediate function, patient and graft survival would improve.

In our study, low molecular weight HES treatment of blood yielded a decline of blood viscosity values. Furthermore it significantly decreased the red blood cell aggregability and slowed the process in time. The aggregate's resistance to dissociation by flow induced shear stress was significantly lower in the HES 130 kDa treated samples when compared to control. The visualization revealed various sized linear rouleaux morphology with a range of 4–7 red blood cells per aggregate.

Questions might arise regarding the efficacy of HES 130 kDa in maintaining the colloid osmotic pressure during the wash–out procedure and preservation period. Hydroxyethyl starches have been used for many years in order to prevent and treat hypovolemia during major surgery: they decrease the transvascular fluid flux and edema formation via maintenance of the colloid osmotic pressure and preservation of the microvascular barrier. In that respect, HES 130 kDa is proved to be an efficacious plasma volume expender in heart surgery[27]. In addition, Zikria et al.[28] demonstrated that 100 to 300 kDa fraction of HES significantly minimized tissue edema in an ischemia–reperfusion model of increased vascular permeability, independent of the colloid osmotic pressure effect. They hypothesized that this finding was related to a biophysical effect of starch effectively sealing the separated endothelial junctions.

Under normal conditions erythrocytes deformability allows individual red blood cell with a mean resting diameter of 7m to traverse capillaries with diameters no more than 3–5 μm. Rigid cells in the postoperative blood flow could present a block in the microcirculatory passageway. Any decrease in the deformability would result in impaired perfusion of organs and peripheral tissues[29,30]. Therefore the present study was designed to investigate the influence of HES on RBC deformability as well. We found no significant effect of HES on RBC deformability ($p>0.05$).

Theoretical models of erythrocyte aggregation

Membrane adhesion processes, including erythrocyte aggregation, can be classified into two categories: specific binding and nonspecific binding. Specific binding occurs via interaction of macromolecules with their specific receptors on the erythrocyte membrane. For nonspecific binding mechanism, two major theoretical models have been proposed[31]. The first theory is based on the surface adsorption of macromolecules to form bridging configuration between adjacent erythrocytes. The adsorption is believed to be favored by Van der Waals forces, hydrogen bounds or electrostatic attractions. According to this theory, polymers and plasma protein with

a large molecular mass insert between adjacent erythrocytes, increase the intercellular distance and induce erythrocyte aggregation by decreasing the electrostatic repulsive forces of erythrocytes[32]. The second theory suggests that the aggregation is induced by macromolecular depletion from the membrane surface. In this theory the aggregation is independent of both the molecular mass and the surface adsorption. The attraction of colloid particles producing the aggregation is induced by variations in the surface energy and differences in osmotic pressure due to a profile of polymer concentration existing in the suspending medium between the neighboring surfaces[33].

Hypotheses on the mechanism of hydroxyethyl starch induced RBC aggregation

Our study documented that the extent of HES induced RBC aggregation varied with the molecular weight. Colloids with high molecular weights such as HES 450 kDa and HES 200 kDa induced a significantly higher aggregation when compared to the physiological aggregation. Concentration of the colloid was shown to be pivotal in the aggregation process. The observed strong correlation of erythrocyte aggregation with the molecular weight and concentration of HES can be explained by the theory of macromolecular bridging. In contrast, the colloid with a small molecular weight, HES 130 kDa, had an inhibiting effect on the extent and kinetics of the aggregation. These findings are consistent with the assumption that inhibition of aggregation occurs because of increase of small molecules in the depletion region.
The study of red blood cell aggregation in a binary HES–HES system showed that both *hyper*-aggregability induced by HES 450 kDa and *hypo*-aggregability induced by HES 130 kDa are reversible phenomena, demonstrating in this way the nonspecific nature of HES adsorption on the surface of the cell.

In summary, our experiments conclusively showed that the physiological function of red blood cells to form aggregates is significantly affected in the presence of hydroxyethyl starch. The aggregation of erythrocytes was extended and accelerated with increasing the molecular weight of HES and its concentration. As a new and unexpected finding, a significantly lower aggregation was observed in HES 130 kDa–treated erythrocytes compared to the aggregation in controls. In addition, the use of a colloid–free UW solution resulted in a complete abolition of RBC aggregability.
The causes of hepatic dysfunction or allograft failure after liver transplantation are multifactorial and identifying risk factors predictive of both patient and graft survival is crucial to improve outcome after transplantation. To date, several risk factors have been shown to negatively affect the graft survival, such as donor/recipient age[34], size of body/weight index, prolonged donor stay in the intensive care unit and long cold ischemic time[35], perfusion during initial wash–out and preservation methods[3]. Most of these factors are static, but some of them are subject to manipulation, for example the use of high molecular weight HES in the formulation of UW solution. We sug-

gest, on the basis of our experimental data, that the use of low molecular weight HES (HES 130 kDa) will improve the quality of the University of Wisconsin solution, have a beneficial effect on organ preservation and possibly reduce the chance of postreperfusion primary nonfunction and posttransplant biliary lesions with delayed recovery in organ transplantation.

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References

1. Clavien PA, Harvey PR, Straßberg SM. Preservation and reperfusion injuries in liver allografts: an overview and synthesis of current studies. *Transplantation.* 1992;53:957-78.

2. Straßberg SM, Howard TK, Molmenti EP, Hertl M. Selection of the donor liver: risk factors after orthotopic liver transplantation. *Hepatology.* 1994,20:829-38.

3. Ploeg RJ , D'Alessandro AM, Knechtle SJ et al. Risk factors for primary dysfunction after liver transplantation – a multivariate analysis. *Transplantation.* 1993;55:807-13.

4. Ploeg RJ, Goossens D, McAnulty LF, Southard JH and Folkert O, Belzer FO. Successful 72-Hour Storage of Dog Kidneys with UW Solutions. *Transplantation.* 1988; 6:191.

5. 't Hart NA, Leuvenink HGD, Ploeg RJ. New solutions in organ preservation. *Transplantation Reviews.* 2002;16:131-141.

6. Nuno J, Cuervas–Mons V, Vicente E, Turrion V, Pereira F, Mora NP, et al. Prolonged graft cold ischemia: a risk factor for early bacterial and fungal infection in liver transplant recipient. *Transplantation Proc.* 1995;27:2323-5.

7. Carrasco L, Sanchez-Bueno F, Sola J et al. Effects of cold ischemia time on the graft after orthotopic liver transplantation. *Transplantation.* 1996;61:393-6.

8. Mor E, Schwartz ME, Sheiner PA et al. Prolonged preservation in University of Wisconsin solution associated with hepatic artery thrombosis after orthotopic liver transplantation. *Transplantation.* 1993;56:1399-402.

9. Adam R, Bismuth H, Diamond T et al. Effect of extended cold ischemia with UW solution on graft function after liver transplantation. *Lancet.* 1992;340:1373-7.

10. Ploeg RJ, van Bockel JH, Langendijk PT et al. Effect of preservation solution on results of cadaveric kidney transplantation. The European Multicentre Study Group. *Lancet.* 1992;340: 129-37.

11. Tojimbara T, Wicomb WN, Garcia-Kennedy R, Burns W, Hayashi M, Collins G, Esquivel CO. Liver transplantation from non–heart beating donors in rats: influence of viscosity and temperature of initial flushing solutions on graft function. *Liver Transpl. Surg.* 1997;3:39-45.

12. Corry WD, Jackson LJ, Seaman GV. Action of hydroxyethyl starch on the flow properties of human erythrocyte suspensions. *Biorheology.* 1983;20:705-17.

13. Chien S. Rheology in the microcirculation in normal and low flow states. *Adv. Shock. Res.* 1982;8:71-80.

14. Bishop JJ, Nance PR, Popel AS, Intaglietta M, Johnson PC. Effect of erythrocyte aggregation on velocity profiles in venules. *Am. J. Physiol. Heart Circ. Physiol.* 2001;280:222-236.

15. Valles J, Santos MT, Aznar J, Marcus AJ, Martinez–Sales V, Portoles M, Broekman MJ, Safier LB. Erythrocytes metabolically enhance collagen-induced platelet responsiveness via increased thromboxane production, ADP release, and recruitment. *Blood.* 1991;78:154-162.

16. Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood.* 1997;89:1121-32.

17. Pearson MJ, Lipowsky HH. Influence of erythrocyte aggregation on leukocyte margination in postcapillary venules of rat mesentery. *Am. J. Physiol. Heart Circ. Physiol.* 2000;279:460-461.

18. van der Plaats A, 't Hart NA, Morariu AM, Verkerke GJ, Leuvenink HG, Ploeg RJ, Rakhorst G. Effect of University of Wisconsin organ–preservation solution on haemorheology. *Transpl. Int.* 2004;17:227–33.

19. Hardeman MR, Goedhart PT, Lettinga KP. Laser-assisted optical rotational cell analyser (L.O.R.C.A.); I. A new instrument for measurement of various structural hemorheological parameters. *Clinical Hemorheology.* 1994;14:605-618.

20. Hardeman MR, Goedhart PT, Lettinga KP. Laser–assisted optical rotational cell analyser (L.O.R.C.A.); I. A new instrument for measurement of various structural hemorheological parameters. *Clinical Hemorheology.* 1994;14:605-618.

21. Osterloh K, Gaehtgens P, Pries AR. Determination of Microvascular Flow Pattern Formation in Vivo. *Am. J. Physiol. Heart Circ. Physiol.* 2000;278:H1142-H1152.

22. Miyagawa Y, Imamura H, Soeda J et al. Fate of hepatocyte and sinusoidal lining cell function and kinetics after extended cold preservation and transplantation of the rat liver. *Liver Transpl.* 2002;8:370-81.

23. Minor T, Hachenberg A, Tolba R, Pauleit D, Akbar S. Fibrinolytic preflush upon liver retrieval from non–heart beating donors to enhance postpreservation viability and energetic recovery upon reperfusion. *Transplantation.* 2001;71:1792-96.

24. Busquets J, Figueras J, Serrano T et al. Postreperfusion biopsy changes predict biliary complications after liver transplantation. *Transplant. Proc.* 2002;34:256-8.

25. Gologorsky E, De Wolf AM, Scott V, Aggarwal S, Dishart M, Kang Y. Intracardiac thrombus formation and pulmonary thromboembolism immediately after graft reperfusion in 7 patients undergoing liver transplantation. *Liver Transpl.* 2001;7:783-9.

26. Suriani RJ, Cutrone A, Cohen E, Konstadt SN, Gabrielseon GV, Miller C. Pulmonary thromboembolism during liver transplantation: Is venovenous bypass protective? *Liver Transpl. Surg.* 1995;1416A.

27. Gallandat Huet RC, Siemons AW, Baus D, van Rooyen–Butijn WT et al. A novel hydroxyethyl starch (Voluven) for effective perioperative plasma volume substitution in cardiac surgery. *Can. J. Anaesth.* 2000;47:1207-15.

28. Zikria BA, Subbarao C, Oz MC, Shih ST et al. Macromolecules reduce abnormal microvascular permeability in rat limb ischemia-reperfusion injury. *Crit. Care Med.* 1989;17:1306-1309.

29. Dormandy JA. Red cell deformability. *Eur. Neurol.* 1983;22:23-29.

30. Stuard J. Erythrocytes rheologgy. *J. Clin. Pathol.* 1985;38:965-977.

31. Brooks DE. Current models of red blood cell aggregation. *Biorheology.* 1995;32:103.

32. Chien S. Biophysical behavior of red cells in suspensions. *The Red Blood Cell.* 1975;2:1032-1133.

33. Evans E, Needham D. Attraction between lipid bilayer membrane in concentrated solutions of nonadsorbing polymers: comparison of mean–field theory with the measurement of adhesion energy. *Macromolecules* 1988;21:1822-1831.

34. Markmann JF, Markmann JW, Markmann DA et al. Preoperative factors associated with outcome and their impact on resource use in 1148 consecutive primary liver transplants. *Transpl. Int.* 1994;7:S672-4.

35. Gruenberger T, Steininger R, Sautner T, Mittlbock M, Muhlbacher F. Influence of donor criteria on postoperative graft function after orthotopic liver transplantation. *Transpl. Int.* 1994;7:S672-4.

Chapter 8

General Discussion

Recent improvements in the clinical care of patients have their roots in two distinct fields of modern medicine: biomedical research and clinical ethics. In order to improve the ability of clinical medicine to apply successfully and ethically the new developments in medical science, research must be undertaken to understand the full effects of medical treatments and the proper threshold for medical intervention. Moreover, it is of critical importance how physicians understand the risks and benefits of treatment and how to guide the decision making for individual patients. The ethical aspects of treatment decisions are of equal importance, with emphasis on patients expectations when they consent to manipulation involving risk factors and on their participation in a treatment decision. Only by investing time and effort in both medical education and research, the ethical ideals that underlie the physician–patient relationship can be fulfilled.

The present thesis describes a generally recognized pathophysiologic mechanism: impairment of organ perfusion with its diagnostic and therapeutic challenges. Out of the multitude of possible etiologic factors for organ perfusion impairment, we chose to investigate two extreme situations of acute organ support: (1) organ perfusion during cardiopulmonary bypass with cardiac arrest and (2) organ perfusion during

organ donation and procurement prior to transplantation. Even if these two clinical fields seemed to be segregated at a first approach, our results conclusively showed a parallelism in etiology, pathologic mechanisms, and therapeutic approaches.

Using this original approach, we investigated several issues of concern for both cardiac surgery and organ transplantation: use of artificial colloids, use of prophylactic corticosteroids, diagnostic value of organ injury markers, consequences of hemodilution, hypothermia induced injury, and vascular endothelial activation.

Effects of hydroxyethyl starches (HES) on red blood cell aggregation

The use of HES solutions as priming and plasma substitution fluids in patient undergoing cardiopulmonary bypass results in altered red blood cell aggregation. In parallel with the decrease in red blood cell aggregation, blood viscosity declines also. The subsequent variations in blood rheology activate the vascular endothelium with pro–inflammatory and pro–thrombotic effects. A distinct effect of different molecular weight starches was evident post–bypass. While the markers of endothelial activation went down to baseline levels in HES 200/0.5 group, HES 130/0.4 was associated on the first postoperative day with sustained endothelial activation.

In organ transplantation, the use of high molecular hydroxyethyl starches (HES 450/0.7 and higher) as components of the University of Wisconsin preservation solution accounts for accelerated and augmented red blood cell aggregation. The aggregates are of large size and their resistance to dissociation by flow induced shear stress is high. These data suggest that gravity–induced hydrostatic perfusion pressures presently used in procurement can not easily dissociate the abnormal red blood cell aggregates. In addition, the small vessel diameter in marginal areas compared to the aggregates size make these vessel category prone to mechanical obstruction during organ procurement. The presence of remaining host erythrocyte aggregates, trapped in the microvasculature after the initial wash–out of the donor organ could contribute to an inadequate microvascular perfusion with preservation solution and therefore to a poor maintenance of graft viability during ischemic storage. The areas of the respective organs that are only marginally equilibrated with University of Wisconsin solution are expected to be less protected during the subsequent ischemic storage period, thus contributing to an overall reduced structural and functional integrity of the organ. Low molecular weight HES treatment of blood yields a decline of blood viscosity values, decreased the red blood cell aggregability and slows the process in time.

Our results documenting the effect of hydroxyethyl starches on red blood cell aggregation suggest the necessity of a more careful selection of HES solutions when

considering a therapeutic strategy. In cardiac surgery, hypertensive and atherosclerotic patients who have already a high basal levels of circulating von Willebrand factor might benefit from HES 200/0.5. HES 130/0.4 could represent a first choice for patients with bleeding tendencies and patients with acquired von Willebrand syndrome after aortic stenosis. In organ preservation prior to transplantation, the exclusive use of low molecular weight HES will improve the quality of the University of Wisconsin solution by preventing intravascular red blood cell aggregation. By preventing mechanical obstruction during wash-out, use of HES 130/0.4 might have a beneficial effect on organ preservation and possibly reduce the chance of postreperfusion primary nonfunction and posttransplant biliary lesions with delayed recovery in organ transplantation.

Prophylactic corticosteroid treatment

The assumption that prophylactic corticosteroid therapy, by its virtue to inhibit the inflammatory response, would also transfer a protective effect of organ injury associated with cardiopulmonary bypass, was rejected by the results presented in this thesis. Dexamethasone treatment offered no protection against transient, perioperative renal, intestinal and hepatic injury in patients undergoing on–pump coronary artery bypass grafting. In fact, dexamethasone treatment seemed to be detrimental, resulting in a pronounced postoperative pulmonary dysfunction, prolonged time to tracheal extubation and by initiating postoperative hyperglycaemia. An important observation was the strong positive correlation found between high blood glucose level, as side effect of dexamethasone, and end organ injury. The necessity of a stricter management of serum glucose emerged, suggesting insulin therapy at serum glucose lower than 10 $mmol.L^{-1}$ (as standard) in order to avoid kidney and intestinal injury. This message is also important for the clinicians responsible for the management of the brain dead organ donors, confronted as well with the use of corticosteroid therapy and glucose management.An early institution of insulin therapy might decrease brain death–related donor organ damage.

Diagnostic value of organ injury markers

With the goal of diagnosing impairment in organ perfusion and subsequent organ injury, the present work investigated in a multitude of clinical settings the use of both standard and newly available organ injury biomarkers. Most of the standard laboratory investigations proved to require long assay times, to lack sufficient specificity and/or sensitivity. In contrary, using newly available, sensitive and specific organ injury biomarkers we were able to document transient, subclinical cardiac, renal, intestinal and hepatic tissue injury even in low risk patients undergoing cardiopulmonary bypass. Similar, these new markers proved to be useful when investigating

early brain death–related donor organ damage.

Fatty acid binding proteins (FABP) are cytosolic proteins with various tissue specific isotypes, released in circulation and subsequently in urine in case of cellular damage. We investigated the use of heart (H), intestinal (I), and liver (L) type fatty acid binding proteins. In cardiac patients undergoing cardiopulmonary bypass, plasma H–FABP correlated with other cardiac injury markers (cardiac Troponin I and creatine kinase MB). The advantage of including H–FABP in the diagnosis of myocardial injury is the early peak arising already one and a half hour after reperfusion, which was significantly earlier than the peaks of cardiac Troponin I (fourteen hours) and creatine kinase MB (sixteen hours). Urinary concentration of H–FABP proved to be a better indication of kidney damage than of myocardial damage, explained possibly by a primary release of H–FABP in urine from the damaged distal renal tubules. In our study on the patients undergoing cardiac surgery, the urinary peak of H–FABP did not correlate with the others cardiac markers but correlated strongly and significantly with the urinary peak of N-acetyl-glucosaminidase (NAG, proximal tubules injury) and peak microalbuminuria (glomerular injury). Similar,in brain dead rats donors, H–FABP and NAG urine concentration reached significantly higher values as early as half hour and one hour, respectively, after brain death induction, as compared with sham operated animals. A highly positive correlation was documented between the two renal tubules markers, consolidating the diagnose of renal tubular damage during brain death.
I/L–FABP are cytosolic proteins readily released into the circulation following enterocytes damage, with a 40-fold higher content of L–FABP, reported as useful urine markers for the detection of intestinal injury. Both urinary I–FABP and L–FABP increased significantly during CPB, reaching peak values in the urine collected during the first two hours and six hours postoperative, respectively. Urine I–FABP correlated significantly with urine L–FABP. The increased values of I–FABP and L–FABP during CPB reported in this study verify the indirect evidence of mucosal integrity loss during CPB reported previously as perioperative reduction in intramucosal pH, increase in gut permeability and endogenous endotoxemia. Using the test of I–FABP concentration in the urine of brain dead rats, we were able to detect intestinal injury as early as two hours after brain death (data not shown).
Additional to fatty acid binding proteins, we would like to emphasize the utility of N–acetyl–glucosaminidase in diagnosing proximal tubules injury, and of α-Glutathione S–transferase in diagnosing hepatic injury.

Consequences of hemodilution

Using a complex operative strategy in patients undergoing on-pump coronary artery bypass grafting, we showed an important attenuation of the transient renal and intesti-

nal postoperative injury achieved by means of limiting intra–operative hemodilution and blood transfusion requirements. Variation in hematocrit explained more than a third of the variation of both postoperative NAG and I–FABP. A decrease with one unit (1%) in hematocrit predicted significantly an increase with a quarter of the peak postoperative NAG values. The same decrease with one unit (1%) in hematocrit predicted significantly an increase with a tenth of the peak postoperative I–FABP values in patients undergoing on–pump cardiac surgery. Hemodilution, besides lowering the oxygen carrying capacity of blood, alters as well blood rheology with possible pathological consequences.

With the aim set on investigating variation in blood rheology during isovolemic hemodilution and subsequent effects on vascular endothelial activation, we designed an animal study to answer previously formulated hypothesis in clinical studies. To bring relevance, the study addressed two different hydroxyethyl starch (HES) solutions commonly used in the clinical practice as priming solutions for the heart–lung machine and as plasma expanders. An important observation was that hemodilution up to 50% resulted in negligible hypoxia/reperfusion injury, as quantified by the reactive oxygen species production measured in the vital organs. Low red blood cell aggregation, as documented in this model of acute isovolemic hemodilution, was associated with activation of vascular endothelium, especially in lungs and small intestine. Translation of these data in clinical terms suggests that acute hemodilution may lead to inflammatory stress of pulmonary capillaries. Subsequent diffusion limitation may be expected. Similar, an increased inflammatory response in the small intestine associated with acute hemodilution, might contribute to a loss in barrier function of the intestinal mucosa with subsequent translocation of endotoxins and/or bacteria. Additionally, the data presented in this study suggest a new pathway for the erythrocyte involvement in clot formation: due to their function to aggregate, erythrocytes can modulate endothelial activation with von Willebrand factor release, with a subsequent pro–thrombotic effect. The investigations on acute isovolemic hemodilution might be clinically relevant for the patients undergoing on–pump cardiopulmonary bypass, the patients in traumatic–hemorrhagic shock with sustained fluid resuscitation, or the brain dead organ donors with large volume of fluid infusion to correct for hypotension. Based on the results demonstrating increased endothelial activation, we hypothesize that lower incidence of thrombotic events and decreased inflammatory reactions could be achieved by avoiding excessive hemodilution.

Hypothermia–related injury

Contrary to conventional thinking about the benefits of corporeal hypothermia on systemic protection against global ischemic injury during extracorporeal circulation, an increasing number of clinical studies support corporeal normothermia. The re-

sults of our clinical investigation comparing normothermia with hypothermia showed a negative correlation between body temperature during cardiopulmonary bypass and postoperative I–FABP urine concentrations. In other words, lower body temperatures during CPB were associated with higher intestinal damage. These findings confirm at a different level, the studies performed in organ transplantation that document a higher organ damage when cold ischemic preservation time is extended. In the clinical setting of organ transplantation, cooling down the organ followed by rewarming is a generally recognized trigger of injury.

Vascular endothelial activation

Pro–inflammatory and pro–coagulatory vascular endothelial activation was demonstrated to be a central pathological finding in both cardiac surgery and brain death organ donation. In cardiac surgery with cardiopulmonary bypass, endothelial activation was demonstrated to arise in the first hours after myocardial reperfusion, as documented by elevated plasma concentrations of von Willebrand factor, thrombomodulin, tissue Plasminogen Activator and E–Selectin. At gene regulation level, endothelial activation is shown in our pig experimental study to arise already three hours after induction of isovolemic hemodilution. In brain dead donor rats, endothelial activation was documented as early as half hour after brain death induction.

The etiology of endothelial activation is multifactorial: systemic inflammatory response, surgical stress, and systemic mobilization of wound–release factors. As original contribution, the present thesis introduces a new etiologic factor: decreased red blood cell aggregation as a trigger of impaired blood rheology and thus mechanical endothelial activation. We hypothesize that the drop in RBC aggregation added to plasma viscosity reduction during hemodilution alone, or even more during extracorporeal circulation, are important factors contributing to variation in shear stress at the vascular endothelial wall. The variation in shear is known to lead to a complex signaling response eventuating in pro-inflammatory and pro–coagulatory vascular endothelial activation.

In conclusion, the work described here aims to add a new foundation stone on the scientific basis for diagnosing and treatment, by contributing to current clinical debates and suggesting new directions for clinical and fundamental research. Additionally, the results included in this thesis emphasize the need of collaborative decision making between physicians with different expertise, and between physicians and researchers.

Perspectives

Development and validation of new extracorporeal assist devices are highly desirable when performing artificial organ support. Pulsatile perfusion remains a challenging therapeutic choice. Either used as bridge to transplantation, bridge to recovery, or during coronary artery bypass grafting with cardiac arrest, an effective pulsatile perfusion might improve clinical outcomes.

In the same line of research, hypothermic machine perfusion providing a pulsatile blood flow is known to offer better protection against cold ischemic injury when compared with cold storage in marginal donor organs. Special effort has to be invested in testing in both experimental and clinical settings the benefits on graft viability when perfused with this newly available hypothermic, pulsatile machine preservation systems.

Last but not least, special scientific attention has to address the pathophysiology of disease and placing it in a clinical relevant context. In this respect, our efforts in documenting new mechanisms of endothelial activation related to variation in blood rheology parameters, the potential consequences of red blood cell aggregation on (micro)circulation might prove to be valuable in managing complications in both cardiac and transplant patients.

Summary

The aim of this work was to investigate the efficiency of organ perfusion during acute organ support, as performed during extracorporeal mechanical blood circulation in cardiac patients, donor management, organ procurement and organ preservation prior to transplantation. The investigations were conducted in clinical studies, animal studies and in-vitro experimental settings. The efforts were concentrated on testing the diagnostic value of new, specific and sensitive biomarkers for organ injury, in order to help an early and effective therapeutic strategy.

Chapter 1 introduces the reader into the subject by offering basic theoretical knowledge concerning cardiopulmonary bypass, organ preservation and organ donation prior to transplantation.

The study presented in **Chapter** 2 was designed to document the effects of dexamethasone on cytokine release and perioperative myocardial, pulmonary, renal, intestinal and hepatic damage, as assessed by specific and sensitive (bio)markers. A prospective, double–blind, placebo–controlled, randomized trial for dexamethasone was conducted in 20 patients, receiving either dexamethasone (1 mg/kg before anesthesia induction and 0.5 mg/kg after 8 hours) or placebo. Different markers were used to asses the inflammatory response: Interleukin–6, Interleukin–8, Interleukin–10, C–reactive protein, tryptase. Organ damage was investigated using plasma heart–type fatty acid binding protein, Troponin I, and Creatine kinase–MB to asses myocardial injury, urine N–acetyl–glucosaminidase and microalbuminuria to investigate renal injury, intestinal/liver type fatty acid binding protein to assess the small intestine injury, and αGlutathione S–transferase for the hepatic injury.
Dexamethasone, as administered in this study, effectively inhibited the release of pro–inflammatory interleukins and increased plasma concentration of anti–inflammatory

interleukins. However, dexamethasone treatment offered no protection against transient, perioperative renal, intestinal and hepatic injury in patients undergoing on–pump coronary artery bypass grafting. Dexamethasone treatment resulted in more pronounced postoperative pulmonary dysfunction, prolonged time to tracheal extubation and initiated postoperative hyperglycaemia. The high blood glucose levels were found strong significant predictors for renal and intestinal tissue injury.

In **Chapter** 3 we described the experimental infrastructure and clinical application of a comprehensive operative strategy that aimed to limit postoperative myocardial, renal and intestinal tissue injury in patients undergoing heart–lung machine assisted coronary artery bypass grafting. A prospective, pseudo–double blind, randomized clinical trial, investigating the clinical benefits of the new experimental intraoperative protocol was performed in 40 patients. The experimental operative protocol was developed to meet multiple objectives: (1) homogeneous cooling of the myocardium by combining cold crystalloid cardioplegia technique with intracavitary cooling of the heart; (2) prevention of excessive hemodilution by autologous priming of the extracorporeal circuit and partial recovery of the cardioplegic fluid; (3) corporeal normothermia, possible on the account of a more efficient topical cooling of the heart. Clinical outcome and transient postoperative injury of the myocardium, kidneys, and small intestine were investigated. Postoperative myocardial damage, as quantified by plasma levels of creatine kinase MB, was significantly lower in the patients in the experimental group. Transient proximal tubules injury was significantly attenuated in the patients benefiting from the experimental operative protocol, as shown by the urine concentrations of N–acetyl–beta–D glucosaminidase. Transient intestinal damage, as quantified by the urinary excretion of intestinal–type fatty acid binding protein, was significantly decreased in patients undergoing on pump coronary artery bypass grafting according the experimental protocol.

With the aim set on investigating variation in blood rheology during cardiopulmonary bypass and subsequent effects on (micro)circulation, we focused in **Chapter** 4 on red blood cell aggregation and endothelial activation. The present study addresses two different hydroxyethyl starch (HES) solutions commonly used in the clinical practice as priming solutions for the heart–lung machine and as plasma expanders. Red blood cell aggregation was measured by means of Laser–assisted Optical Rotation Cell Analyzer, in an in vitro study designed to mimic the human blood–material interactions during extracorporeal circulation. A clinical study investigating endothelial activation was conducted in 20 patients undergoing elective coronary bypass surgery, randomly assigned for cardiopulmonary bypass using either HAES–steril 6% (HES 200/0.5) or Voluven 6% (HES 130/0.4).
The property of red blood cells to form aggregates at low shear rates was profoundly altered in our in vitro model mimicking the human blood–material interactions during

extracorporeal circulation. In parallel with the decrease in red blood cell aggregation, blood viscosity declined also. A functional and/or structural alteration of vascular endothelial cells during extracorporeal circulation was documented by elevated plasma concentrations of von Willebrand Factor, thrombomodulin, tissue plasminogen activator and E–Selectine. Differences between HES groups were evident post–bypass. While the markers of endothelial activation recovered in HES 200/0.5 group, HES 130/0.4 was associated on the first postoperative day with further increase of vWF and tPA.In parallel with the decrease in red blood cell aggregation, blood viscosity declined also. A functional and/or structural alteration of vascular endothelial cells during extracorporeal circulation was documented by elevated plasma concentrations of von Willebrand Factor, thrombomodulin, tissue plasminogen activator and E–Selectine. Differences between HES groups were evident post–bypass. While the markers of endothelial activation recovered in HES 200/0.5 group, HES 130/0.4 was associated on the first postoperative day with further increase of vWF and tPA.
The observations made in vitro on red blood cell aggregability coupled to the observation made in vivo on endothelial cell activation suggest a hypothetical new pathophysiological mechanism implicated in the post–cardiopulmonary bypass syndrome. We hypothesized that the drop in red blood cell aggregation added to plasma viscosity reduction and non–physiologic flow conditions during extracorporeal circulation, are important factors contributing to variation of shear stress at the vascular wall, leading to endothelial activation.

In **Chapter** 5, the hypothesis emerged in the previous chapter, concerning a possible interconnection between red blood cell aggregation and endothelial function, is verified in an experimental animal model of isovolemic hemodilution.We induced acute isovolemic hemodilution (30 ml/kg exchange transfusion with colloid solutions) in an "aggregating species" (pigs), and investigated the hypoxic oxidative stress (*plasma Malondialdehyde, ex–vivo oxygen radicals production in heart, lung, kidney, liver, ileum tissue–biopsies*), erythrocyte aggregation (LORCA), and endothelial activation (Real Time Quantitative Taqman RT–PCR on von Willebrand Factor (vWF), E– and P–Selectins, and endothelial nitric oxide synthase gene–expression in tissue biopsies). The production of superoxide and hydroxyl radicals, measured as H_2O_2 generation, was similar at all times in sham–operated and hemodiluted animals, which indicates that a similar hypoxic oxidative stress is present, and oxygen delivery to the tissue during acute hemodilution is maintained. Acute isovolemic hemodilution was followed by a dramatic drop in erythrocyte aggregation and immediate pro–thrombotic (significant vWF mRNA up–regulation in heart, lungs, kidney, liver, ileum) and pro–inflammatory (significant E– and P–Selectins mRNA up–regulation in lungs and ileum) endothelial activation. Low erythrocyte aggregations were significantly associated with increased mRNA–expressions of vWF (heart, liver, ileum) and P–Selectin (lungs, ileum and heart). In this way, we were able to demonstrate that

erythrocyte aggregation can actively modulate thrombogenicity and inflammation by inducing release of endothelium–dependent pro–thrombotic factors and expression of pro–inflammatory adhesion molecules.

Approaching the subject of organ perfusion in transplantation, the study presented in **Chapter** 6 we investigated the time sequence for the progression of pro–inflammatory and pro–coagulatory endothelial activation, oxidative stress and organ injury in brain dead rat donors. The brain death model used in this study was a slow model, simulating the clinical condition of brain death due to intracranial hemorrhage. Brain death was induced by slowly inflating a balloon–catheter inserted in the extradural space. To assess time–dependant changes due to brain death, rats were sacrificed half hour, one hour, two hours, and four hours after brain death induction and compared to sham–operated controls.
The mRNA expression of the adhesion molecules E– and P–Selectins, known to promote inflammation by mediating rolling and extravasation of leukocytes, were up–regulated shortly (half hour) after brain death induction. Additionally, platelet trapping, most probably due to platelet adhesion to the vascular wall, was visualized as early as half hour after inducing the brain death. A significant increase in plasma levels of von Willebrand factor, and increased mRNA expression of Aα and Bβ fibrinogen, were observed.
Oxidative stress started to increase after induction of brain death, and became significant only after two hours of brain death. Brain death related donor kidney damage was diagnosed as early as half hour in renal tubules, with enhanced loss of viability when the state of brain death was prolonged.

When assessing the efficiency of organ procurement, the study in **Chapter** 7 aimed to determine the effect of HES and University of Wisconsin solution on the extent and kinetics of human red blood cell aggregation, and to morphologically characterize these aggregates. Human red blood cell aggregability and deformability were investigated in vitro, at 4° C, with a Laser–assisted Optical Rotation Cell Analyzer. The study of red blood cell aggregation in a binary HES–HES system gave an indication about the nature of HES–red blood cells interactions. Bright field microscopy and atomic force microscopy were used to morphologically characterize the aggregates size and form.
High molecular weight HES and University of Wisconsin solution had a potent hyper-aggregating effect. red blood cell aggregates were of large size and their resistance to dissociation by flow induced shear stress was high. These data suggest that gravity–induced hydrostatic perfusion pressures presently used in procurement can not easily dissociate the abnormal red blood cell aggregates. In addition, the small vessel diameter in marginal areas compared to the aggregates size make these vessel category prone to mechanical obstruction during organ procurement. Low molecular weight

HES treatment of blood yielded a decline of blood viscosity values, decreased the red blood cell aggregability and slowed the process in time.

Perspectives

Development and validation of new extracorporeal assist devices are highly desirable when performing artificial organ support. Pulsatile perfusion remains a challenging therapeutic choice. Either used as bridge to transplantation, bridge to recovery, or during coronary artery bypass grafting with cardiac arrest, an effective pulsatile perfusion might improve clinical outcomes.

In the same line of research, hypothermic machine perfusion providing a pulsatile blood flow is known to offer better protection against cold ischemic injury when compared with cold storage in marginal donor organs. Special effort has to be invested in testing in both experimental and clinical settings the benefits on graft viability when perfused with this newly available hypothermic, pulsatile machine preservation systems.

Last but not least, special scientific attention has to address the pathophysiology of disease and placing it in a clinical relevant context. In this respect, our efforts in documenting new mechanisms of endothelial activation related to variation in blood rheology parameters, the potential consequences of red blood cell aggregation on (micro)circulation might prove to be valuable in managing complications in both cardiac and transplant patients.

Samenvatting

Dit proefschrift beschrijft enkele experimentele en klinische onderzoek naar vroegtijdige diagnostiek van orgaanschade. Met als doel een bijdrage te leveren aan een optimale bescherming van organen in situaties waarin de normale bloedcirculatie verstoord is.

Hoofdstuk 1 verschaft de lezer inzicht in de theoretische kennis op het gebied van cardiopulmonary bypass, orgaan preservatie en orgaan donatie voor transplantatie.

Hoofdstuk 2 Omdat tijdens hartoperaties met behulp van een hart–long machine (cardiopulmonary bypass) een ontstekingsreactie wordt opgewekt, werd altijd aangenomen dat corticosteroïden een beschermende werking zouden hebben; immers, corticosteroïden remmen ontstekingsreacties. Talloze studies hebben inderdaad een remming van de ontstekingsreactie door corticosteroïden tijdens hartoperaties aangetoond, maar niet eerder is met gevoelige biochemische meetmethoden de specifieke orgaanschade bestudeerd. Hoofdstuk 2 beschrijft een studie waarbij éénmalig het corticosteroïd dexamethason werd toegediend aan een groep patiënten vlak voordat een hartoperatie werd uitgevoerd. Een vergelijkbare (controle) groep patiënten kreeg geen corticosteroïden. Dexamethason bleek geen bescherming tegen nier–, darm– en leverschade te bieden. Dexamethason bleek zelfs enkele nadelige gevolgen voor de patiënt te hebben aangezien er een slechtere long functie direct na de operatie werd geconstateerd, waardoor de kunstmatige beademing langer duurde. Daarnaast bleek dat de bloedsuiker spiegel direct na de operatie sterk verhoogd was. Met name deze sterk verhoogde bloedsuiker spiegel wordt verantwoordelijk geacht voor de orgaanschade. De onverwachte nadelige gevolgen van dexamethason konden mede verklaard worden door de remming van tryptase, een enzym dat vrijkomt uit mestcellen tijdens cardiopulmonary bypass. Dit enzym biedt mogelijk bescherming tegen orgaanschade; tryptase gaat vaatvernauwing tegen welke juist optreedt door verhoogde bloedsuiker

spiegels en door de verstoorde doorbloeding tijdens cardiopulmonary bypass. Deze studie pleit daarom, met name bij gebruik van corticosteroïden, voor het tijdig toedienen van insuline om de schade door te hoge bloedsuiker spiegels te voorkomen of in elk geval te verminderen.

Hoofdstuk 3 Verdunnen van het bloed en het verlagen van de bloedtemperatuur zijn twee methoden die vanaf het begin van het gebruik van een hart–long machine zijn toegepast om het lichaam te beschermen tijdens hartoperaties. Hoofdstuk 3 beschrijft onze studie waarbij het bloed van de patiënt zo weinig mogelijk wordt veranderd. Hiermee bedoelen we het volgende; in het medisch centrum van de Vrije Universiteit (VUMC) is een operatiestrategie ontwikkeld, waarmee het bloed nauwelijks verdund wordt en alleen het hart gekoeld wordt. Daartoe wordt de canule (waardoor het bloed tijdens de operatie vanuit de patiënt naar de hart–long machine stroomt) zodanig aangepast dat het tevens een koelsysteem voor het hart bevat. Het hart wordt hierdoor beter gekoeld, terwijl het lichaam bijna de normale temperatuur blijft behouden. Bloedtransfusies worden hiermee beperkt, aangezien het bloed minder verdund wordt. Met behulp van deze nieuwe methode bleek de schade aan hart–, nier– en darmweefsel in belangrijke mate beperkt te worden.

Hoofdstuk 4 Deze studie toont aan dat met het gebruik van bepaalde plasma vervangende middelen de doorstroming van weefsels en de stolling van het bloed sterk beïnvloed kan worden. Voorafgaand aan een hartoperatie wordt de hart–long machine gevuld met een plasma vervangend middel, in totaal ongeveer 1,5 tot 2 liter. Hierna wordt de hart–longmachine aangesloten aan de bloedcirculatie van de patiënt, waarna het bloed en de plasma vervangende vloeistof worden vermengd. Plasma vervangende middelen kunnen de eigenschappen veranderen van de rode bloedcellen (erythrocyten) om te aggregeren. Dit aggregeren is een natuurlijk proces dat dient om het transport van erythrocyten door de bloedcirculatie te verbeteren. In de grotere bloedvaten vormen erythrocyten dergelijke aggregaten. Maar deze aggregaten moeten volledig uit elkaar vallen zodra de erythrocyten de haarvaten zullen gaan passeren. De molecuulgrootte van plasma vervangende middelen blijkt dit aggregeren te beïnvloeden en daarmee de manier waarop de erythrocyten door de bloedvaten stromen. In dit hoofdstuk wordt een vergelijkende klinische studie beschreven waarin een plasma vervanger HES 200 (grote moleculen, 200kD) vergeleken wordt met HES 130 (kleine moleculen, 130 kD). HES 200 heeft aggregerende eigenschappen die lijken op die van normaal plasma. HES 130 daarentegen verlaagt de aggregatie van erythrocyten en verlaagt daarmee de stroperigheid (viscositeit) van het bloed.
Daarenboven wordt de aggregatie van erythrocyten verlaagd door de bloedverdunning die tegelijkertijd optreedt. Hoofdstuk 4 beschrijft verder nog het vrijkomen van ontstekings– en stollinsproducten uit de bloedvaatwand (endotheel) tijdens gebruik van HES 130. De veronderstelling is dat dit veroorzaakt wordt door een verander-

de snelheidsgradiënt langs de vaatwand, waardoor endotheel–activatie plaatsvindt. Aanvullend fundamenteel en klinisch onderzoek zijn nodig om dit vraagstuk verder te ontrafelen.

Hoofdstuk 5 Hierin wordt een studie beschreven die bij varkens is uitgevoerd. Het bloed (ongeveer 2 liter) van het varken werd vervangen door een HES 200 of HES 130 oplossing. Aansluitend werd weefselschade gemeten. Zoals verwacht bleek dat de erythrocyten aggregatie sterk verminderd was na deze bloedverdunning, maar onverwacht werd in dit varkensmodel geen verschil aangetoond in beïnvloeding van de erythrocyten aggregatie tussen HES 200 en HES 130. De zuurstof afgifte in de weefsels bleek na verdunning nog steeds voldoende te zijn. Er werd echter een sterke stijging van stollingsproducten en ontstekingsfactoren vanuit endotheelcellen aangetoond in alle onderzochte organen (darmen, nier, lever, long en hart). Vooral in de abdominale organen (darmen, nier, lever) werden grote veranderingen gemeten. Een afname van complicaties kan dus bereikt worden door ernstige bloedverdunning te voorkomen, hetgeen de bevindingen uit eerder genoemde klinische studies (Hoofdstuk 3 en 4) onderbouwt.

Nu we enig inzicht hebben gekregen in de orgaanschade bij verstoorde circulatie en acute bloedverdunning en de rol van endotheelcellen daarin, is het interessant te weten hoe groot de orgaanschade is indien er sprake is van ernstige afwijkingen in de bloedcirculatie en endotheel activatie zoals bij orgaantransplantaties gebeurt. De volgende twee hoofdstukken behandelen enkele van deze aspecten.

Hoofdstuk 6 Organen van patiënten met fataal hersenletsel hebben in een aanzienlijk aantal gevallen een tegenvallende orgaanfunctie. Een van de oorzaken daarvan is de verandering (verslechtering) in de bloedcirculatie die optreedt na hersenschade. In Hoofdstuk 6 wordt een studie bij ratten beschreven, waarin het optreden van orgaanschade in de tijd gemeten werd na het aanbrengen van hersenschade. Al een half uur na hersendood werd in het bloed een toename gemeten van stollingsfactoren en ontstekingsfactoren. Na 1 uur was ook schade aan de nier meetbaar, na 2 uur was de productie van zuurstof radicalen verhoogd. De conclusies van deze studie suggereren dat bij orgaandonoren het tijdig toedienen van middelen om de stolling en ontsteking tegen te gaan orgaanschade zou kunnen beperken.

Hoofdstuk 7 Om o.a. stolselvorming in een donor orgaan te voorkomen, moet het goed doorgespoeld worden. Daardoor kan de bewaarvloeistof goed doordringen in het weefsel. Bewaarvloeistoffen bevatten plasma vervangende middelen om oedeem te voorkomen. De meest gebruikte bewaarvloeistof, genaamd University of Wiconsin Solution, bevat het plasma vervangende middel HES 450. In het onderzoek beschreven in Hoofdstuk 7 wordt aangetoond dat de grote moleculen van HES 450 de aggregatie

van erythrocyten zodanig versterkt, dat de aggregaten bijna niet meer uit elkaar vallen en zo groot zijn dat de kleinere bloedvaten verstopt raken. Hierdoor is het niet mogelijk de donor organen goed te doorspoelen en kan na transplantatie een deel van het weefsel zelfs afsterven. Vervangen van HES 450 door HES 130 zou een logische methode kunnen zijn om het genoemde probleem op te lossen.

Toekomstvisie

Ontwikkeling en validatie van nieuwe circulatie ondersteunende apparatuur zijn erg belangrijk voor het handhaven van orgaanfuncties tijdens het ontbreken van normale hartslag. Het onderzoek van het nut van pulserende circulatie blijft een uitdaging. De kennis op het gebied van orgaanfunctie kan aangewerd worden om de effecten van pulserende circulatie nauwkeuriger te onderzoeken. Dit kan het klinische eindresultaat verbeteren tijdens overbrugging naar transplantatie, om het hart tijdelijk te ontlasten, of tijdens een hartoperatie waarbij het hart stil staat.

Uit hetzelfde onderzoeksgebied is bekend dat machinale pulsatiele vloeistof circulatie van een donororganen beter beschermt tegen weefsel schade dan statische koude preservatie, waarbij het orgaan in ijs verpakt wordt. Het is van groot belang de voordelen van deze nieuwe pulsatiele preservatie–machines te bepalen in zowel de experimentele als klinische omgeving teneinde een groter aantal donor organen met succes te kunnen transplanteren.

Tot slot, extra wetenschappelijk aandacht is nodig om de onderliggende oorzaken van ziektes te onderzoeken. Onze inspanningen in het beschrijven van nieuwe mechanismen van endotheel–activatie, gerelateerd aan veranderingen in de stromingseigenschappen van bloed, zouden zeer waardevol kunnen zijn voor een goede behandeling van patiënten met (tijdelijke) circulatie stoornissen, zoals hart– en transplantatiepatiënten.

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LIST OF PUBLICATIONS

1. *Morariu, A.M.*; van der Plaats, A.; van Oeveren, W.; 't Hart, N.A.; Leuvenink, H.G.D.; Graaff, R.; Ploeg, R.J.; Rakhorst, G.; Effects of Hydroxyethyl Starch on Human Red Blood Cell Aggregability. Am. J. Transpl., 2(3), 347, 2002.

2. *Morariu, A.M.*; van der Plaats, A.; van Oeveren, W.; 't Hart, N.A.; Leuvenink,H.G.D.; Graaff, R.; Ploeg, R.J.; Rakhorst, G.; Hyperaggregating Effect of HES Components and University of Wisconsin Solution on Human Red Blood Cells: A Risk of Impaired Graft Perfusion in Organ Procurement? Transplantation, 76, 37-43, 2003.

3. van den Eijnden, M.M.E.D.; Leuvenink, H.G.D.; Ottens, P.J.; 't Haart, N.A.; van Oeveren, W.; *Morariu, A.M.*; van Goor, H.; Ploeg, R.J.; Effect of brain death and non–heart–beating kidney donation on renal function and injury: an assessment in the isolated perfused rat kidney. Experimental and Clinical Transplantation, 2, 85-89, 2003.

4. van der Plaats, A.; 't Hart, N.A; *Morariu, A.M.*; Verkerke, G.J.; Leuvenink, H.G.D.; Ploeg, R.J.; Rakhorst, G.; Effect of University of Wisconsin organ–preservation solution on haemorheology. Transpl. Int., May 14, 2004.

5. van den Eijnden, M.M.E.D.; Leuvenink, H.G.D.; Ottens, P.J.; 't Haart, N.A.; van Oeveren, W.; *Morariu, A.M.*; van Goor, H.; Ploeg, R.J.; Effect of brain death and non-heart-beating kidney donation on renal function and injury. American Journal of Transplantation 4: 391-391 854 Suppl. 8 2004.

6. *Morariu, A.M.*; Gu, Y.J.; Galladat Huet, R.C.G.; Siemons, W.; Rakhorst, G.; van Oeveren, W.; Red Blood Cell Aggregation During Cardiopulmonary Bypass: A Pathogenic Cofactor in Endothelial Cell Activation? Eur. J. Cardiothorac. Surg. 2004 Nov;26(5):939-46.

7. *Morariu, A.M.*; Loef, B.G.; Aarts, L.P.H.J.; Rietman, G.W.; Rakhorst, G.; van Oeveren, W.; Epema, A.H. Prophylactic use of dexamethasone in cardiopulmonary bypass. Chest 126 (4): 854S-854S Suppl. S OCT 2004.

8. *Morariu, A.M.*; Loef, B.G.; Aarts, L.P.H.J.; Rietman, G.W.; Rakhorst, G.; van Oeveren, W.; Epema, A.H.;Dexamethazone: Benefit and Prejudice for Patients Undergoing On–pump Coronary Artery Bypass Grafting Accepted for Chest 2005.

9. *Morariu, A.M.*; Huybregts, M.A.J.M.; Rakhorst, G.; Burgerhof, J.G.M.; de Vroege, R.; van Oeveren, W.; Combined Strategy to Limit Perioperative Myocardial, Renal and Intestinal Tissue Injury in Patients Undergoing On–pump Coronary Artery Bypass Grafting. Accepted for The Journal of Thoracic and Cardiovascular Surgery 2006.

10. *Morariu, A.M.*; Schuurs, T.A.; Leuvenink, H.G.D.; van Oeveren, W.; Rakhorst, G.; Ploeg, R.J.; Pro–Coagulatory and Pro–Inflammatory Endothelial Activation in Brain Dead Donors. Submitted to Transplantation.

11. *Morariu, A.M.*; Maathuis, M.H.J.; Asgeirsdottir, S.A.; Leuvenink, H.G.; Boonstra, P.W.; van Oeveren, W.; Ploeg, R.J.; Molema, G.; Rakhorst, G.; Acute Isovolemic hemodilution triggers pro-inflammatory and pro-coagulatory endothelial activation in vital organs: role of erythrocyte aggregation. Submitted to Cardiovascular Research.